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Carbonic Anhydrase Inhibition. I. The Pharmacology of Diamox® 2-Acetylamino-1,3,4-Thiadiazole-5-Sulfonamide.
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CARBONIC ANHYDRASE INHIBITION

I: The Pharmacology of Diamox® 2-Acetylamino-1,3,4-Thiadiazole-5-Sulfonamide*

THOMAS H. MAREN,[1] EDMUND MAYER AND BARBARA C. WADSWORTH

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* The initial phases of this work were presented November 10, 1952, at a monthly meeting of the New York Academy of Sciences (Biology Section) and published in Tr. New York Acad. Sc., Series II, 1952, **15**: 53.

[1] Formerly Instructor in Pharmacology and Experimental Therapeutics, The Johns Hopkins University School of Medicine.

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Introduction

Diamox 2-acetylamino-1,3,4-thiadiazole-5-sulfonamide (6063, acetazoleamide; Fig. 1) is a specific inhibitor of the widely distributed animal enzyme, carbonic anhydrase. It is the first compound of this type to be introduced into medicine. This and the following two papers (15, 30) describe the pharmacology, analytical methods, and renal effects that pertain to Diamox. As an introduction, the present section will attempt a brief history of carbonic anhy-

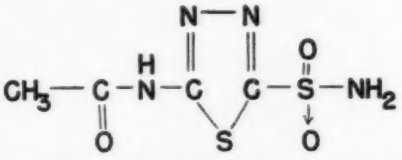

Fig. 1. Structure of Diamox® 2-acetylamino-1,3,4-thiadiazole-5-sulfonamide (6063, acetazoleamide).

drase and its inhibitors (Table I), including the physiological activities of the enzyme (Table II) and the bases for the use of its inhibitors in the treatment of certain diseases (Table III).

Present knowledge indicates that carbonic anhydrase has a single chemical role, catalysis of the rate of attaining equilibrium in the reaction

$$H_2O + CO_2 \underset{\text{anhydrase}}{\overset{\text{carbonic}}{\rightleftharpoons}} H_2CO_3 .$$

The secondary equilibrium $H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$ is instantaneous, so that the enzyme-sensitive reaction is the rate-controlling event in the overall process. Equilibria may be readily approached from either side, as illustrated by *in vitro* techniques which utilize either the formation of H^+ from CO_2 and water, or the evolution of CO_2 from HCO_3^- [reviewed in (17)].

Biological reactions in this system are entirely similar. Table II indicates the nature and probable locus of carbonic anhydrase activity in various tissues. For details see the reviews by Davenport (16) and Van Goor (17) and the more recent papers cited in Table II and Table III. No role has been discovered for carbonic anhydrase in brain. Its distribution in the central nervous system has been thoroughly studied (37).

The present work originated from the studies listed in Table I. It is of interest to recall that the metabolic effects of sulfanilamide (2, 2a, 3, 9) were accurately described before its action on this enzyme was known, and that

TABLE I

Studies Leading to the Present Investigation

1932	Discovery of Carbonic Anhydrase. Meldrum and Roughton (1)
1937	Sulfanilamide Produces Acidosis, Loss of Fixed Base, and Rise in Urinary pH. Southworth (2, 2a); Marshall *et al.* (3)
1940	Further Studies of Sulfanilamide on Acid-Base Metabolism. Beckmann *et al.* (9)
1940	Sulfanilamide and Other N^4 (Amino) Substituted Sulfonamides as Carbonic Anhydrase Inhibitors. Mann and Keilin (4)
1941	Carbonic Anhydrase Found in Kidney. Davenport and Wilhelmi (10)
1942	Effect of Sulfanilamide Acting as an Inhibitor of Carbonic Anhydrase in Raising the pH of Frog Urine. Höber (5)
1945	Renal Effect of Sulfanilamide, i.e. Decrease in Acid Excretion, Shown in Dogs; Urinary Acidification Related to Hydration of CO_2, Catalyzed by Carbonic Anhydrase. Pitts and Alexander (6). See also (6a)
1949	Effect of Sulfanilamide as Diuretic in Congestive Heart Failure. Schwartz (7)
1950	Synthesis of Diamox (6063) and Other Heterocyclic Sulfonamides, with Increased and Specific *In Vitro* Activity Against Carbonic Anhydrase. Roblin *et al.* (11, 12)
1951	Acute Renal Effect of Diamox in Dog, with Particular Respect to K^+ Excretion. Berliner *et al.* (14)

TABLE II

Carbonic Anhydrase Activity in Mammals*

Tissue	Product Separated	From Tissue To
Erythrocyte (1)	CO_2	Air
Kidney: tubules ? (5, 6)	H^+	Urine
Stomach: parietal cells (23)	H^+	Gastric Juice
Pancreas: acini ? (26)	HCO_3^-	Pancreatic Juice
Eye: ciliary body (29)	HCO_3^-	Aqueous Humor

* Expressed in the reaction $H_2O + CO_2 \xleftarrow[\text{anhydrase}]{\text{carbonic}} H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$.

these puzzling metabolic effects provided the stimulus for Mann and Keilin's (4) discovery that aromatic sulfonamides *unsubstituted* on the $—SO_2NH_2$ nitrogen are carbonic anhydrase inhibitors. This relationship still holds (8, 12); the substituted N^1 sulfonamides which followed sulfanilamide into antibacterial chemotherapy are virtually devoid of inhibitory activity against carbonic anhydrase. As Mann and Keilin suggested, substitution or loss of the $—NH_2$ nitrogen of sulfanilamide and related compounds do not abolish inhibitory activity; more recently it appears (8, 12) that substituents on the N^4 (amino) nitrogen actually enhance activity. The specificity of the aromatic $—SO_2NH_2$ group for carbonic anhydrase is notable; no other type of compound with more than 10% of the activity of sulfanilamide has been described (61).

The immediate advantage of 2-acetylamino-1,3,4-thiadiazole-5-sulfonamide over sulfanilamide was that of activity, the new compound having inhibitory activity *in vitro* 60–330 times that of sulfanilamide, depending on the system

TABLE III

Current Research on Diamox

Subject	Author	Physiological Basis	Result
Electrolyte Effect in Man	Bergstrom, *et al.* (28)	Inhibition of Renal Carbonic Anhydrase	Reduction in renal H^+ secretion. Initial loss of base and sustained acidosis on Diamox q.6 h.
Electrolyte Effect in Man	Nadell (18)	Inhibition of Renal Carbonic Anhydrase	Reduction in renal H^+ secretion. Initial loss of base and sustained acidosis on Diamox q.6 h.
Pulmonary Effect in Man	E. L. Becker, *et al.* (19)	Inhibition of Blood Carbonic Anhydrase	No change in arterial or alveolar CO_2 or gradient 3 hours after single dose.
Pulmonary Effect in Anesthetized Dogs	Tomashefski, *et al.* (20)	Inhibition of Blood Carbonic Anhydrase	Retention of CO_2 following single high doses of Diamox.
Diuresis in Congestive Heart Failure	Friedberg, *et al.* (21)	Inhibition of Renal Carbonic Anhydrase	Diuresis and maintenance of about 75% of moderately to mildly ill cardiacs, single or multiple doses.
Diuresis in Congestive Heart Failure	Belsky (22)	Inhibition of Renal Carbonic Anhydrase	Diuresis and maintenance of about 75% of moderately to mildly ill cardiacs, single or multiple doses.
Diuresis in Congestive Heart Failure	Relman, *et al.* (60)	Inhibition of Renal Carbonic Anhydrase	Ineffective in severely ill hospitalized cardiacs.
Electrolyte and Clinical Effect in Respiratory Acidosis	Nadell (18)	? Probably secondary to inhibition of renal enzyme and loss of base. Also decrease of lung edema ?	Lowering of pCO_2 and clinical improvement in two patients. Multiple daily dose.
Inhibition of Gastric HCl in Dog	Janowitz, *et al.* (23)	Inhibition of Gastric Carbonic Anhydrase	Reduction of HCl secretion by 85% in the Heidenhain pouch. Reduction of HCl in gastric segment.
Inhibition of Gastric HCl in Dog	Rehm, *et al.* (24)	Inhibition of Gastric Carbonic Anhydrase	Reduction of HCl secretion by 85% in the Heidenhain pouch. Reduction of HCl in gastric segment.
Inhibition of Gastric HCl in Man	McGowan and Stanley (25)	Inhibition of Gastric Carbonic Anhydrase	Reduction of HCl secretion in normals and ulcer patients, after high single doses.
Inhibition of Pancreatic Secretion in the Dog	Birnbaum and Hollander (26)	Inhibition of Pancreatic Carbonic Anhydrase	Reduction of HCO_3^- secretion into pancreatic duct.
Reduction of Intraocular Tension in Glaucoma	B. Becker (27)	Inhibition of Ciliary Carbonic Anhydrase	Reduction of tension, with clinical relief. Presumably, lowering of HCO_3^- secretion.

TABLE III—*Continued*

Subject	Author	Physiological Basis	Result
Treatment of Epilepsy in Children	Bergstrom *et al.* (28)	? Acidosis Secondary to Inhibition of Renal Carbonic Anhydrase? or Inhibition of Brain Carbonic Anhydrase ?	"Clinical improvement in 8 of 42 patients previously intractable." Multiple daily doses.
Effect of Diamox in Elasmobranch	Heinemann and Hödler (46)	Absence of Response to Alkali by Elasmobranch Kidney	Acidification of urine in elasmobranch not inhibited by Diamox, and does not involve a carbonic anhydrase system. Blood pCO_2 rose, indicating effect on gas transport.
Effect of Diamox in Alligator	Hernandez and Coulson (47)	Urinary Excretion of Ammonium bicarbonate by Alligator	Diamox decreases urinary HCO_3^- but not NH_4^+, with resulting systemic alkalosis.

This Table includes publications through June, 1954.

used[2] (12). The basis for selection of this compound (then called 6063) for further study over other highly active heterocycles was its ready oral absorption and lack of *in vivo* destruction (13). The potent effects of intravenous 6063 as a renal carbonic anhydrase inhibitor in acute experiments was reported by Berliner *et al.* (14). The compound was found to have strikingly low acute and chronic toxicity, and high renal activity by the oral route.* Details of the renal (mammalian) effects of Diamox (6063) are described in a succeeding paper (15). In summary, inhibition of this enzyme diminishes tubular secretion of H^+ (see Table II), which then leads to excretion of HCO_3^-, Na^+, K^+, and H_2O.

The clinical usefulness of carbonic anhydrase inhibitors in increasing excretion of sodium in congestive heart disease was first recognized by Schwartz in his studies on sulfanilamide (7). With the development of an inhibitor more active and less toxic than sulfanilamide, clinical evaluation in this disorder was begun (21, 22, 60). Current research on Diamox, relating both to its renal and extra-renal effects, is listed in Table III; some non-mammalian effects are also included.

[2] In the system more recently used (30) this ratio is 64, but may vary widely, depending on details of the test. If less substrate (CO_2) and less enzyme are used, for example, the ratio is almost 1000.

* Recent work suggests that 300 mg./kg. of sulfanilamide has the same *renal* activity (approximately one third of maximal base loss for this type of agent) in the dog as 3 mg./kg. of Diamox. Since the renal handling of these drugs is similar (partial tubular reabsorption), this 100/1 ratio may be a reasonable index of their relative activity.

The present paper is concerned with the pharmacology of Diamox in experimental animals, and, to a lesser extent, in man. We have arbitrarily set off as a succeeding publication (15) the detailed effects of this drug on acid-base metabolism. Since these effects are intimately related to the fate of the drug, there are numerous cross-references between the two papers; it will be noted that many of the animals used for toxicity and absorption work were simultaneously studied with respect to acid-base balance. There were many advantages of this arrangement, particularly since the chief and possibly the only toxic effects of Diamox in animals were associated with distortions of electrolyte balance.

About two-thirds of the present study concerns the absorption, distribution, excretion and toxicity of Diamox. In addition to these traditional pharmacological studies, the remainder of this paper is devoted to the particular properties of this drug as a carbonic anhydrase inhibitor. In view of the ubiquitous role of carbonic anhydrase in the body, the number of facets that demand study is clearly beyond the scope of a single laboratory. These special studies carry unequal weight, and indication will be made to the reader of the number of experiments available for each question, so that the "degree of reliability" may be appraised.

Methods and Materials

Beagles that have been raised and bred in this laboratory for the past 12 years were used. The rats were from Carworth Farms, of a modified Wistar strain. Albino mice and guinea pigs were also used for acute experiments. Experiments with Rhesus monkeys were carried out at Lederle Laboratories through the courtesy of Dr. H. Koprowski and Mr. J. Black.

Dogs were sacrificed by intravenous injection of pentobarbital. The autopsies included examinations of the cranial, thoracic and abdominal cavities and the femoral marrow. Organ samples were fixed in formalin and/or Bouin's fluid. The adrenals were preserved also in potassium dichromate formalin. In some cases the whole lungs were preserved in 70% alcohol for histologic and chemical study of calcium deposits. Paraffin sections were made in all cases and in addition frozen sections were made of liver and kidneys. Rats were sacrificed by intraperitoneal injection of pentobarbital. *Complete* autopsies of rats were analogous to those performed in the dogs. In *abbreviated* autopsies of rats the organs, except for femoral marrow, were inspected grossly; liver and kidneys were weighed, but no microscopical examinations were made.

Blood glucose was determined by the Folin-Malmros method. Renal excretion of phenol red was determined by alkalinization of urine followed by dilution and reading at 550 mμ on a Beckman Spectrophotometer. Creatinine was determined by the Jaffe reaction (31). Standard clinical procedures were followed for examination of urine and blood. Hemoglobin values are expressed in millimols O_2 capacity/liter blood. The normal values in the beagles of our laboratories are 8–10 millimols. This is equivalent to 15 g. hemoglobin/100 cc. blood.

The method for the determination of Diamox and of carbonic anhydrase in biological material is described in the following paper (30). This method measures activity rather than the chemical itself, but we have shown (see Section II below) that this activity in dog urine agrees quantitatively with drug isolated, crystallized, and identified from such urine.

Analytical methods for electrolytes, as well as the electrolyte composition of food, are described in a succeeding paper (15).

Diamox (11, 12) is a white crystalline solid which melts at 258–259°C. (corrected). M.W. 222.24. It is very weakly acidic, pK_a 7.2. At 20°C. approximately 50 mg. dissolves in 100 ml. water. For oral use in animals, capsules were made up (dogs), or the drug was incorporated into the diet (rats). In some cases, suspensions in water were given by stomach tube.

The free acid may be suspended in water and injected subcutaneously or intraperitoneally into animals. For intravenous use, however, the acid is dissolved by the addition of 1.6 equivalents of sodium hydroxide. Five and 10% solutions thus prepared have a pH of approximately 9. Such solutions are stable for several weeks at room temperature, and for at least 7 months at 4°C.

Experimental

I. In vitro activity of Diamox on carbonic anhydrase

The high activity of Diamox against blood carbonic anhydrase (12) was confirmed. In the system described (30), Diamox was 64 times as active as sulfanilamide, using 50% inhibition of the enzyme as criterion. Similar activity was demonstrated against renal and gastric carbonic anhydrase.

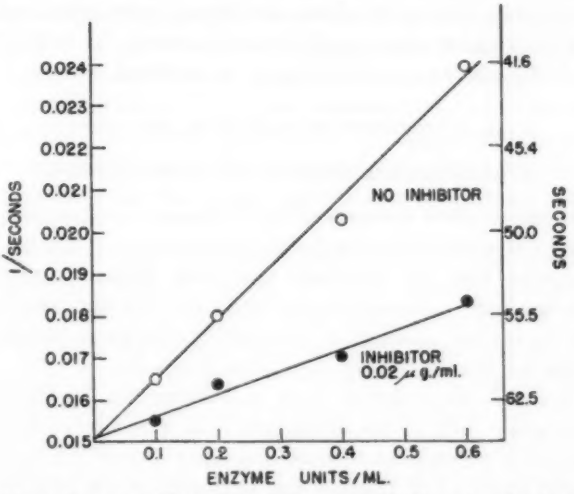

Fig. 2. Evidence for reversible nature of Diamox inhibition of carbonic anhydrase, according to criteria of Ackermann and Potter (32). Enzyme purified by method of Mann and Keilin (4).

Enzyme activity determined by method described in the accompanying paper (30). The upper line (open circles) shows the effect of decreased amounts of enzyme on the reaction time in the system $CO_2 + H_2O \rightleftharpoons H_2CO_3$.

The lower line (solid circles) portrays the effect of a similar decrement in enzyme, but in the presence of constant concentration of Diamox. Since these lines intersect, it is evident that carbonic anhydrase activity is demonstrable with minimum quantities of enzyme, even in the presence of inhibitor, i.e. there is free enzyme present.

At the suggestion of Dr. R. O. Roblin, Jr., the question of reversibility of carbonic anhydrase inhibition by Diamox was studied. Recognition of reversibility (32) depends on reaction rates obtained when various enzyme concentrations are used, without inhibitor (open circles in Fig. 2) and with a constant amount of inhibitor (solid circles in Fig. 2). If inhibition is reversible, the lines through these two sets of points are of different slope and intersect. Figure 2 (see also Legend) indicates that these criteria are fulfilled by the system under discussion.

The *in vitro* activity of Diamox is not altered by heating the drug for 5 minutes at 100°C., either in the presence or absence of tissue. The activity is not reversed by *p*-aminobenzoic acid or by zinc salts. Homogenates of rat tissues, heated to 100°C. for 5 minutes to destroy carbonic anhydrase activity, do not affect the rate of either the catalyzed or uncatalyzed hydration of CO_2 in this procedure.

When the enzymatic method is used for pharmacological work, as described in the accompanying paper (30), no attempt is made to equilibrate drug and enzyme. As indicated in that procedure, the steps are carried through as rapidly as possible, and under these conditions, good reproducibility is attained. In special cases, when equilibration is desired (as in Fig. 7), this has been found to be complete in 3 minutes at room temperature.

II. DISPOSITION OF DIAMOX IN THE BODY

A. Concentrations in Blood and Urine; Clearances

Figures 3a and 3b show concentrations of Diamox in blood and recovery in urine in a dog following 20 mg./kg. by the intravenous (a) and by the oral (b) route. Absorption from the intestinal tract was judged complete within 2 hours. This has been confirmed many times for this and smaller doses. At higher doses by mouth, absorption is somewhat erratic, as judged by urinary excretion data for 33 and 100 mg./kg. doses (Table X).

Figures 3a and 3c show that at doses of 5–20 mg./kg. by the oral or intravenous route about 70% of the unchanged drug is recovered in the urine. In ten experiments in which dogs received oral doses of 3–20 mg./kg., an average of 73% (range 54–91%) of Diamox was recovered in the 24-hour urine. One male and eight females were used; one female was used twice. By "recovery" we again mean as assayed by the enzymatic method (15), which has been shown to agree with gravimetric determination of the isolated drug (Table VII).

The duration of the effect on urinary flow and pH is indicated; this effect persists so long as plasma concentrations of Diamox are over 2 micrograms/ml. This relationship is more fully discussed in a succeeding paper (15) which in-

cludes further data on plasma concentrations of drug at various doses. Diamox appears to remain in erythrocytes long after it disappears from the plasma.

Figure 3c also gives the measurements of simultaneous clearances of Diamox and of creatinine performed by the usual techniques. It appears that clearance of Diamox is roughly two-thirds that of creatinine. This figure was also found at the higher dose level of 33 mg./kg. of Diamox (Schedule of Table X). Presumably it is filtered through the glomeruli and reabsorbed by the tubules. Calculation of the volume of distribution of Diamox from these experiments gives a value of approximately 40%.[3]

In the long term treatment of dogs with Diamox, to be described below (Table X), the plasma concentration of Diamox did not build up. Each day following dosage this value was a fairly accurate reflection of the dose (Table IV), and declined to low or zero values by the next day. In contrast, the Diamox in red cells reached a concentration that persisted around the clock and was largely independent of dose in the ten-fold range studied.

Table V shows the concentrations of Diamox in the blood of rats receiving drug in their diet. As in the dog, plasma concentrations are roughly proportional to dose, and the erythrocytes have a fairly fixed concentration of drug, regardless of dose or accompanying plasma concentration. In the rat, at 10 mg./kg. by any route, the 24-hour urinary recovery is strikingly less than that in the dog, averaging about 30% of the administered dose. The full data are given in connection with electrolyte studies (15). Higher oral doses up to 1000 mg./kg. gave similar recoveries, in agreement with the evidence of Table V that absorption from the diet is not compromised by increase of dose in this species.

In man, there are certain quantitative differences to those described above. In the two patients shown in Fig. 4a, *all* of the administered Diamox could be found in the urine within 24 hours after dosage. In similar studies done in twelve other patients, the average figure was 90%, with the lowest 66%. This is higher than the figure cited above for urinary recovery in the dog. The approximate volumes of distribution in man are indicated in Figure 4b and Table VI. This figure is calculated as shown in Table VI, assuming complete absorption and no destruction. Distribution volumes in man appear somewhat lower than that of dog, and plasma concentrations after due corrections for dose tend to be somewhat higher and more prolonged in man. Table VI shows some of the pharmacologic sequelae to continued daily oral dosage in man. The same cycle of blood concentration and urinary recovery occurred on each

[3] Preliminary studies of protein binding of Diamox by Dr. R. R. Roepke in this laboratory indicate that at drug concentrations of 5–20 micrograms/ml. in plasma, approximately 70–80% of Diamox is unbound.

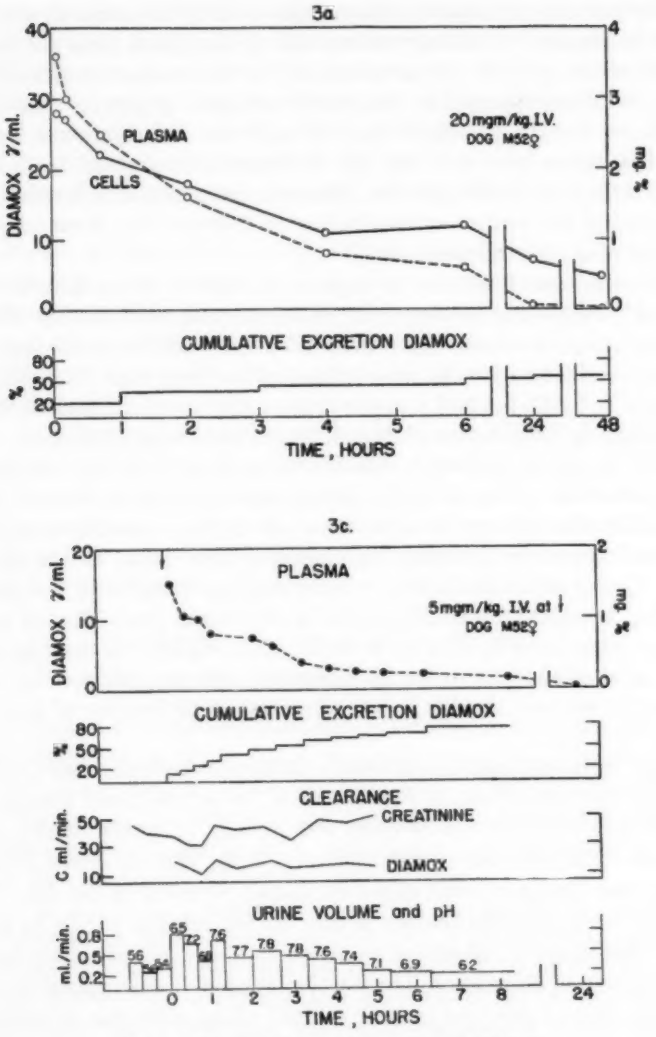

FIG. 3. Pharmacology of Diamox in adult dogs.

a. Drug given as 5% solution of sodium salt.

c. Received 500 cc. H_2O 16 hours before test. Creatinine (4 g.) given subcutaneously 90 minutes before 0 time. Drug given as 2% solution of sodium salt.

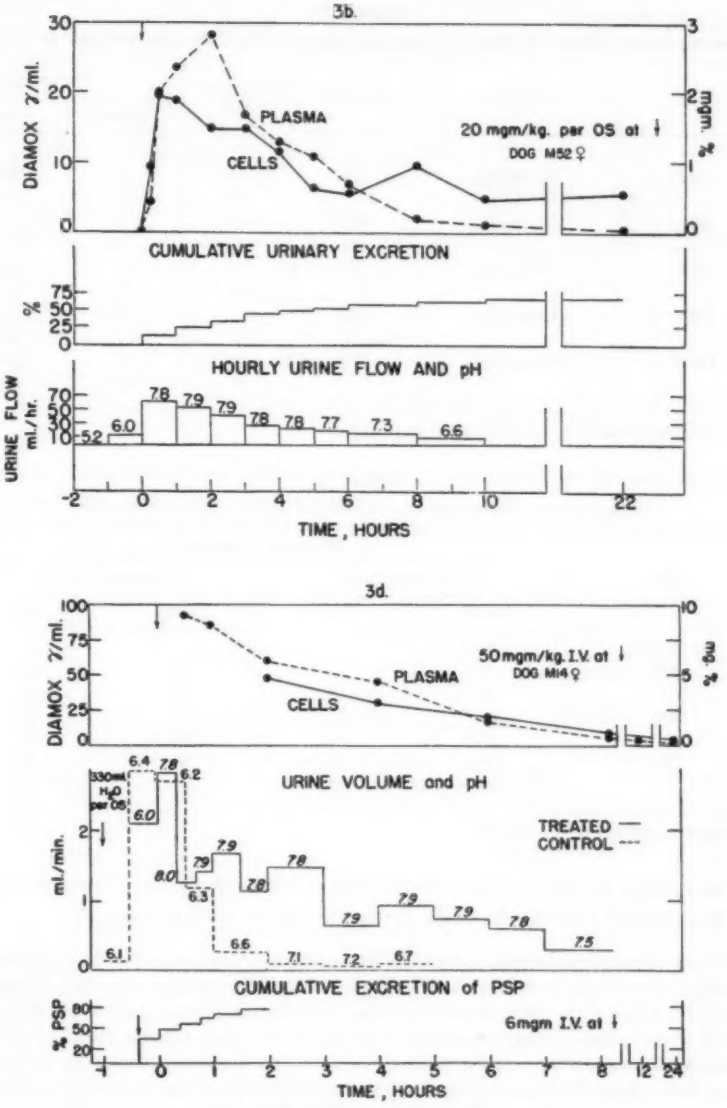

FIG. 3—*Continued*

b. Drug given as 1% aqueous suspension. Water withheld.

d. Drug given as 10% solution of sodium salt. Water diuresis induced 1 hour before 0 time, on both control (-----, no drug) and treatment (———) days. PSP = phenolsulfonphthalein (phenol red).

TABLE IV

Concentration of Diamox in Blood of Adult Dogs During Long-Term Administration of Diamox

Dog	Daily Dose	Day of Exp.*	Hrs. After Dose	Diamox	
				Plasma	Cells
				γ/ml.	
A42	10 mg./kg.	63	0	0	10
			6	3	12
P21	33 mg./kg.	162	0	0	10
			6	7	14
M78	33 mg./kg.	162	0	1	10
			6	9	13
P27	100 mg./kg.	280	0	2	12
			6	37	29
M73	100 mg./kg.	280	0	0	10
			6	18	20

* For schedule of drug administration see Table X.

TABLE V

*Concentration of Diamox in Plasma and Red Cells of Rats on Drug Diet**

Dose	Micrograms Diamox/ml.			
	1 Month from Onset		2 Months from Onset	
	Plasma	Cells	Plasma	Cells
mg./kg./day				
100	4	—	2	22
	2	28	3	23
300	10	30	9	20
	15	—	15	23
900	28	34	30	28
	35	37	45	44

* Experiment of Figs. 5a and 5b.

of the 6 days of observation spaced over a 3-month period. There is clearly no tendency of Diamox to build up in plasma or erythrocytes.

In both dog and man there is often seen an inexplicable plateau in the time-plasma concentration curve, usually at low plasma concentrations. For the dog, such data is noted in Figures 3a, c and d. An indication of a similar phenomenon in man is shown in Figure 4a. During these periods, urinary excretion proceeds at the usual rate, so that the constant plasma level must reflect a redistribution of Diamox in the body, or possibly a chemical change in the drug. This irregularity denotes merely a plateau of activity, not indicating the nature of the active compound. However, since about 70% of Diamox is excreted unchanged in the dog (by the criterion of isolation, see next section), the fraction of drug which might be altered in such fashion is relatively small.

The activity represented by these small "plateau" concentrations in the dog (about 1–2γ/ml.) is less than the activity associated with typical renal response to Diamox; i.e., at such low concentrations reacidification of the urine is observed after the phase of alkaline urine which follows a single dose [Figs. 3b and 3d, and (15)]. Thus, we are confronted only

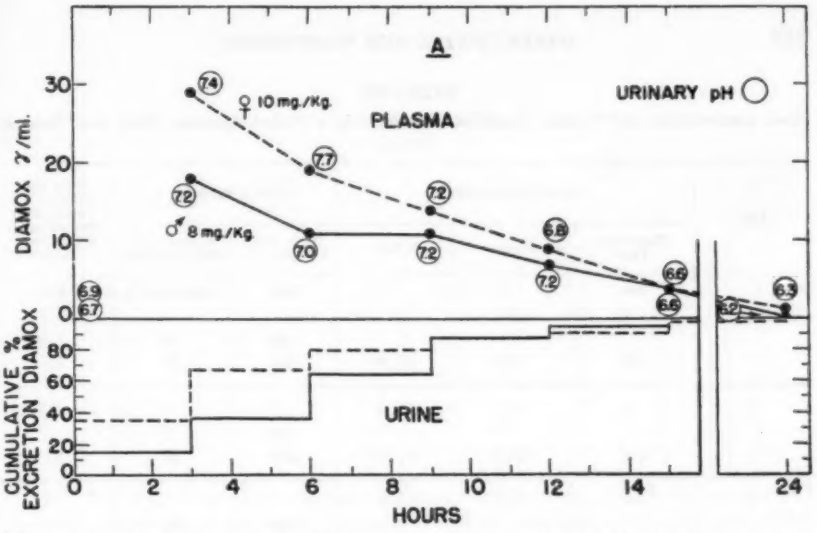

FIG. 4a. Oral administration of Diamox in man, 500 mg. (= 2 tablets) taken at 0 time. Two patients with chronic cirrhosis of the liver.

These patients were under treatment at the Hospital of the Rockefeller Institute for Medical Research, by Dr. William Eisenmenger.

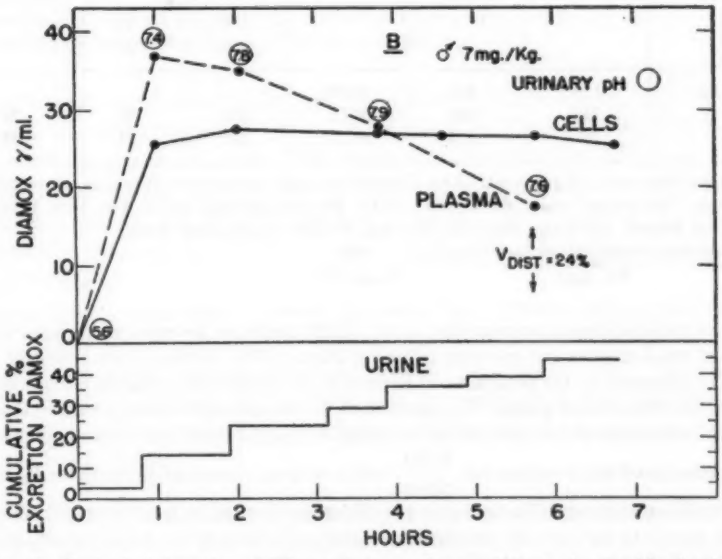

FIG. 4b. Oral administration of Diamox in normal man, 500 mg. (= 2 tablets) taken at 0 time. $V_{dist.}$ is approximate volume of distribution, calculated on the basis of the plasma concentration and drug remaining in the body at 6 hours; for calculation see Table VI, footnote.

Note that the fate of the drug is not substantially different in the normal and in these cirrhotic patients.

TABLE VI

Blood Concentration and Urinary Excretion of Diamox in a Patient Receiving Daily Oral Doses of 250 mg.

Day	Blood Concentration			Urinary Excretion		Drug Distributed in a Volume Corresponding to X% of Body Weight*
	Time After Dose	Plasma	R.B.C.	Time After Dose	Excretion of Previous Dose	
	min.	*γ/ml.*		*min.*	*Cumulative %*	*X%*
1	0	0	0			
				89	4	
	197	8.7	21.0	208	21	32
2	0	1.9	12.8			
				64	2	
	194	13.8	37.0	206	20	20
3	0	2.3	15.5			
				80	10	
	188	12.8	26.3	199	21	22
9	0	1.2	7.0			
				93	13	
	227	6.5	22.0			
37						
				75	10	
				196	26	
83	0	2.1	10.0			
	210	9.0	21.5	210	21	31
	282	6.5	16.0	282	31	37

Patient Ra, male, 54 years, wt. 72 kg. Chronic bronchial asthma and advanced pulmonary emphysema. This patient was under the care of Dr. Morton Galdston at the New York University Research Service, Goldwater Memorial Hospital, Welfare Island, New York.

* $\frac{\text{Amount of drug left in body (mg.)}}{\text{Wt. (kg.)}} \times \frac{100}{\text{plasma level}}$.

with an unusual plasma concentration curve, which would not be expected to influence the cycle of renal activity and recovery following Diamox (15). Neither is the course of renal activity influenced by the persistence of Diamox in the erythrocyte, which is of much greater magnitude than that of plasma. The question of whether persistent plasma and erythrocyte concentrations are related deserves further study. It may be noted that after administration of unsubstituted sulfonamides, the $\frac{\text{R.B.C.}}{\text{plasma}}$ ratios of drug concentration are generally higher than 1; the substituted sulfonamides now in clinical use have ratios less than 1 (55). Whether this is related to the carbonic anhydrase inhibitory properties of the former group, and the presence of the enzyme in red cells, remains to be studied.

B. Isolation of Diamox from Urine

Data in the above section indicate that following Diamox administration, an active carbonic anhydrase inhibitor was found in the urine, and depending

TABLE VII

Recovery of Diamox from the Urine of Dogs

Dose and Route	Actual Drug Received	Vol. Urine	Time	Dose Recovered in Urine, %		% of Dose Lost in Filtrates During Isolation Process, as Determined by Assay
				Assay	Isolation*	
	g.	*cc.*	*hours*			
1000 mg./kg. per os	13	260	24	9†	7	2
500 mg./kg. I.V.	4.5	580	24	69	43	
1000 mg./kg. I.V.	12	550	6	50	43	5
		940	6–24	32	10	16
		1490	Total 0–24	82	53	21
1000 mg./kg. I.V.	12	510	6	43	41	3
		540	6–24	27	18	7
		1050	Total 0–24	70	59	10

* By J. W. Clapp, according to the following procedure: The sludge (if any) was separated by filtration or centrifugation, and saved. The pH of the filtrate was adjusted with conc. hydrochloric acid to ca. 4. After overnight refrigeration, the precipitate was collected, combined with the first crop, and dissolved in dilute base. The solution was clarified with Darco, then acidified to ca. pH 4, with chilling. The precipitate was filtered, washed, and dried.

In every case this product was substantially pure Diamox, as demonstrated by a melting point of 251° (cor.) with decomposition, as compared to the m.p. of 254–255° (cor.) with decomposition for a standard sample; the mixed m.p. was not depressed. Infrared absorption spectra (determined in these Laboratories, under the direction of Dr. R. C. Gore) were identical.

† Reflects incomplete absorption at this dose. See text.

on the species, amounted to 30–100% of the administered dose, calculated as Diamox. The experiment of Table VII shows that Diamox is identical with the inhibitor assayed in urine. The assay and the isolation procedure check closely on the 6-hour samples, where drug concentration was high and loss in the filtrate low. On the 24-hour samples, if the figures for assay of the filtrates be added to those of the isolated Diamox, they approximately equal the total found by assay.

From these experiments it seems apparent that Diamox is excreted in the urine of dogs largely unchanged, and that the material analyzed in biological fluids by the assay method described (30) is the original drug. We believe that in the dog a fraction (30%) of Diamox is metabolized to an inactive (not a carbonic anhydrase inhibitor) substance, and in the rat a large (about 70%) fraction is so altered.

C. Concentration in Body Fluids: Aqueous Humor, Cerebrospinal Fluid, Gastric Juice and Milk

Table VIII gives the results of several experiments in which penetration of Diamox into the aqueous humor and cerebrospinal fluid were studied. It seems clear that in the various conditions and species studied, the $\frac{\text{CSF}}{\text{plasma}}$ and

TABLE VIII

Concentrations of Diamox in Cerebrospinal Fluid (CSF) and Aqueous Humor

Species	Dose and Route	Time After Dose	Diamox Concentration	
			Plasma	CSF
		hours	*γ/ml.*	
Dog P31	20 mg./kg. oral	1	18	0.4
		3	—	0.4
		4	11	0.5
		6	4	0.2
		24	1	<0.05
Dog M70	500 mg./kg. oral at 9 A.M. and 5 P.M. Samples on 5th day after A.M. dose	2.5	44*	3.0
Dog M70	1000 mg./kg. oral daily	2	58	2.6
			Plasma	Aqueous
Cat	38 mg./kg. intravenously	1	44	5.8
Rabbit†	25 mg./kg. intravenously and 25 mg./kg. intraperitoneally	2	12	0.6
Rabbit†	10 mg./kg. intravenously	0.5	8.5	0.5

* At this very high dose, plasma concentration of Diamox was found to be reasonably constant (±9%) in the 1–5 hour period after dose. Absorption is incomplete, being about 10% of the dose.
† Ref. (59).

$\frac{\text{aqueous humor}}{\text{plasma}}$ ratios of this drug are low. The two ratios are similar, roughly 0.05. Plasma binding of Diamox has but a small influence[3] on this figure; a large moiety of unbound Diamox in plasma fails to reach either cerebrospinal or aqueous fluids. Further work with more attention to achievement of equilibrium may be expected to yield more precise figures, although this state was probably achieved in at least one of the experiments of Table VIII (dog M70, 500 mg./kg.).

The following are preliminary data on the appearance of Diamox in gastric juice and milk. Following the intravenous injection of 30 mg./kg. of Diamox into a vagally denervated gastric pouch dog,[4] the drug appeared in the gastric juice within 2 minutes at a concentration of 10 micrograms/ml.; during the subsequent 4 hours of observation the concentration declined but slightly. Reference to Figure 3a indicates that this is about half the concentration that would be expected in plasma at the midpoint of the experiment.

During the period of long-term administration of Diamox to dogs, to be described below, one of the animals [M73 (Table X)] was mated and went on to deliver two pups at term, neither of which survived. The mother treated with Diamox [M73] was then used as a source of milk for new-born dogs from an untreated mother, since it was planned to expose the pups to Diamox from birth (Fig. 6). The dosage schedule of M73 was 100 mg./kg./day by mouth,

[4] Samples kindly supplied by Dr. Henry Janowitz of Mt. Sinai Hospital, New York.

which she received for a total of 16 months. Following this dose, her milk contained the following concentrations of Diamox: at 4 hours, 27 micrograms/ml.; at 8 hours, 20 micrograms/ml.; and at 24 hours, 10 micrograms/ml. This decay curve is strikingly different from that of plasma, but indicates considerable drug in the milk, not unlike the pattern seen in the gastric juice. The nursing puppies had barely detectable levels of Diamox (about 1 microgram/ml.) in their plasma and 8 micrograms/ml. in their erythrocytes. Their progress is shown in Figure 6, and will be discussed below (Section III, B, 2b).

D. Distribution in Organs after Long-Term Treatment

At the termination of the toxicity studies in rats, which will be described below, Diamox was determined in their tissues. Table IX gives the results. The concentrations found in the erythrocytes agree with those of Table V; these are the highest consistent values found in any tissue. Kidneys are next highest; since this organ is excreting the drug, concentrations there should reflect dose and plasma level. Such is the case. The erratic findings for thyroid and pancreas cannot be explained. Localization of the drug, with respect to its effect on the enzyme in any tissue, will probably not be revealed by these gross methods. The cells containing the enzyme and responsible for the ionic exchanges in question may be, on a weight basis, a negligibly small fraction of the whole organ.

TABLE IX

*Tissue Concentrations of Diamox (μg. Diamox/g. Wet Tissue) in Rats Following Drug Diets for 6 Months**

Organ	Dose, mg./kg.				Organ	Dose, mg./kg.			
	100		300			100		300	
	Rat 28 Male	Rat 62 Female	Rat 19 Male	Rat 9 Male		Rat 28 Male	Rat 62 Female	Rat 19 Male	Rat 9 Male
Adrenals	2	6	2	1	Lung	4	2	3	1
Brain	0	1	0	2	Skel. Muscle	6	0	3	0
Brown Fat	5	10	3	6	Ovary		2		
Conn. Tissue	8	8	3	4	Pancreas	24	1	12	2
Fem. Marrow	8	12	14‡		Spleen	5	4	9	4
Heart	3	5	2	0	Testes	0		0	2
Hypophysis		3	14‡		Thyroid	5	0	37‡	
Kidney	10	9	29	27	Yellow Fat	1	6	6	4
Liver	2	4	5	2	Plasma†	1	0	3	
					Erythrocytes†	31	34	24	

* Animals were sacrificed at 10 A.M.–1 P.M., a time about 6–9 hours after the period of maximum food intake. They were exsanguinated following pentobarbital anesthesia. As far as possible, samples of about 1 g. wet weight were obtained. The dissecting microscope was used for removing fat and connective tissue. Particular care was taken to separate the diffuse pancreatic tissue from fat. Wet weights were obtained immediately after dissection.

† From different rats than used for tissue analysis, but in the same groups and identically treated from the start.

‡ From the two rats combined.

In general, the values of Table IX are low in view of the high doses given. This shows that Diamox does not accumulate in tissues.

III. TOXICOLOGY OF DIAMOX

A. Single Dose: Various Species and Routes

Eighteen experiments were done in which intravenous doses of the sodium salt of Diamox, ranging from 2 to 2000 mg./kg., were injected into dogs. Thirteen different dogs were used. None of them died. A male dog that received 2000 mg./kg., in a volume of 80 ml. over a 70 minute period, showed the following signs. There was deep respiration, ataxia, copious urination, and thirst. Later in the day there was tendency to sleep, with continued unsteadiness of gait. Only this latter sign persisted to the following day, and by the third day there was complete recovery. Higher doses were not tried; at 2000 mg./kg. the injection included 180 meq. Na^+ and this cation itself in such amounts (about 40% of total body store) is not devoid of toxicity. An intravenous dose of 80 mg./kg. in an unanesthetized dog produced no change in blood pressure, respiration, or electrocardiogram.

Mice were also given Diamox intravenously. The LD_{50} was between 3000–6000 mg./kg. In comparison with other sodium salts, Diamox appeared about equal to the chloride, but somewhat less toxic than the lactate.

All of seventeen rats survived single oral doses of 1000 mg./kg. All of six rats survived subcutaneous doses of 1500 mg./kg., but half of this number died at 3000 mg./kg. Four guinea pigs survived 750 mg./kg. subcutaneously; two out of six died at 1500 mg./kg.

These tests establish Diamox as a compound with remarkably low acute toxicity. More important, however, are data from continued dosage, which are given below.

B. Multiple Doses

1. Short-Term Experiments: High Doses, Intravenous and Oral. Dog. Continued oral doses in the dog at 500 and 1000 mg./kg./day for 1–2 weeks produce some weakness, anorexia, and vomiting. However, as noted above, these doses are not completely absorbed; at the 1000 mg./kg. level absorption falls off to about 10% of the oral dose.

Two dogs received 100 mg./kg./day intravenously for 10 days, with symptoms similar to those produced by larger doses. During the last 6 days of this experiment plasma pH averaged 7.1, potassium 2.3 meq./l., and sodium 130 meq./l., with no suggestion of further change. During this time food was refused. Urinary excretions of sodium and potassium were small after the first few days of the experiment (15), but in the absence of food intake, such losses, added to the initial base excretion, could become significant. At the

end of the 10-day period there was a net loss of 56 and 62 meq. of sodium and 206 and 161 meq. of potassium in each of the dogs. Since the dogs were moderately depleted of base and rejected food, this dosage schedule may be regarded as seriously toxic, although not lethal. Administration of parenteral sodium (24 meq.) and potassium (64 meq.) chlorides on the ninth and tenth day of this test in one of the dogs appeared to produce some improvement. These added salts were not excreted.

It was evident that higher doses would be necessary to produce death in the dog in a short time. Accordingly, two adult female dogs (P35 and P36) were given 1000 mg./kg. intravenously each day. (This of necessity included approximately 90 meq. of sodium at each injection.) Certain electrolyte determinations which were done during this period indicate the sequence of events, which ended fatally for both dogs at the end of the third day. In the first 24 hours of this schedule they lost 86 and 109 meq. of potassium with no net loss or gain of sodium. Presumably this depletion of potassium continued, for on the third day their plasma potassium was 1.5 and 0.8 meq./l. On this day both animals were in coma and they died in the 10–23 hour period after the third dose. If the dogs lost as much potassium on each of the 2 succeeding days as they did on the first (15), they would have lost about 60% of their total exchangeable potassium (which would be about 500 meq. in these 12-kg. dogs). It was concluded, on the basis of the plasma concentration and the renal excretion of potassium, that these dogs died of severe and acute loss of this ion.

Autopsy findings in these dogs were unremarkable except for the following: One animal (P35) had considerable concretions of Diamox in the renal pelvis, ureter, and bladder. Drug was identified by melting point (J. W. Clapp) and infrared spectroscopy (R. C. Gore). The same animal, on microscopic examination of the liver, showed pronounced stagnation of bile in the capillaries. Both animals showed venous congestion of the liver and kidneys.

Apart from this experience, lethal depletion of potassium has not been observed either in the dog or in other species. Two mechanisms that prevent lethal depletion are suggested by our more detailed studies on electrolyte excretion after Diamox, and are summarized as follows: (a) If *small* doses are given every 24 hours so that losses of base are confined to a 6-hour period (as in Fig. 3c), the animals are able to replace such loss by retention of potassium (and sodium) in the subsequent hours and before receiving the next dose. (b) If *high* doses are given every 24 hours, such a recovery period is shorter (as in Fig. 3d) and the animal cannot replace its initial loss of base. It therefore becomes acidotic, shows a sodium and potassium deficit, and is refractory to further challenge with Diamox. In either of these contrasting situations it is protected against lethal depletion of base. Explanation of the fatal cases cited just above appears to lie in the fact that on moderate to high

doses of Diamox and with good food intake there is generally net loss of potassium for the first 3 days of treatment but not thereafter (15). Daily doses of 1000 mg./kg., combined with the large amount of sodium injected, appeared to cause continuous high output of potassium for a 3-day period. Our total experience indicates that when an animal survives this critical period and eats normally, it is out of danger. Protection is then afforded by either of the mechanisms (a or b) described earlier in this paragraph.

Monkey. Two monkeys were given 100 mg./kg. intravenously for 14 days. They appeared normal throughout. Two other monkeys were given 500 mg./kg. intravenously every 12 hours for 3 days; one showed slight weakness and anorexia; the other appeared normal. By comparison with the experiments cited above on dogs, there is the suggestion that Diamox is less toxic to the monkey than to the dog. It can be questioned whether the extent of potassium losses in the dog can apply to other species, including man, since the canine has strikingly less potassium in red cells (8 meq./l.) than man or monkey (*circa* 100 meq./l.) (33); thus red cells in the dog may provide little reservoir against renal loss. This is more significant in view of the fact that dog erythrocytes are least permeable to potassium of any species studied (34).

2. Long-Term Experiments. a. Adult Dogs. (1) *Observations during Life.* Five dogs were intensively studied over a 16-month period. Representative pharmacological, hematological, and electrolyte data are shown in Table X.[5] During the first few weeks of treatment, the dogs on the highest (100 mg./kg./day) dose showed deep respiration, some vomiting, and occasional muscular fibrillation. These signs were accompanied by anorexia and depression. Although the symptoms resulting from this dose proved reversible, they extended past the period of increased initial potassium excretion, and included days (as day 17) when plasma potassium was not markedly low. A possible clue to the loss of appetite is the fact that potassium deficiency may cause intestinal distention and paralytic ileus (35), and it has recently been shown that plasma concentrations of potassium would not necessarily be correlated with this phenomenon (36). The potassium deficit in these high-dose dogs was estimated at approximately 100 meq., or 15% of the body potassium (15). In the recent studies of Fourman (56), loss of 28% total potassium was produced by resin treatment in man, with symptoms very much like those observed here. Fourman also observed that these were worse in the presence of acidosis, and like ourselves, he cannot decide at present what role potassium

[5] These were selected at appropriate intervals from the larger sequence of test days. In many cases several measurements were also made throughout the day; these did not vary appreciably from the 24-hour (after dose) values entered. Such variations are included in data of the electrolyte paper (15).

loss plays, and what role acidosis, in the symptoms noted. The signs of anorexia and depression observed in the dog may also be a direct effect of Diamox on the central nervous system, where there is carbonic anhydrase in considerable concentrations (37). These signs following Diamox appear restricted to the dog at high doses, and do not seem to have a counterpart in the rat or monkey even at much higher doses (500–1000 mg./kg.). The sleepiness which has occasionally been observed in man (21, 22) did not occur in any of the animals.

These phenomena entirely disappeared after 5 weeks of treatment. For the next 6 months the dogs appeared entirely well. Urine was examined at intervals for albumin, sugar, and casts, and found normal. Blood NPN and plasma calcium and phosphorus levels, determined at intervals throughout treatment, were normal (Table XII). As indicated, the acidosis persisted throughout treatment. One dog on high dosage (M73) went through a pregnancy which appeared normal regarding term and behavior. However, both offspring died in the first week after birth. This will be discussed below. During the seventh month of treatment the second dog (P27) on the high dose began to lose appetite and weight. On day 213 her plasma potassium was 2.40 meq./l. and she weighed 8.4 kg. She was given Purina dog chow checkers, which have high potassium content (see 15), and which she would invariably prefer to other food. Ten days later potassium concentration had risen to 3.08 meq./l. and she had gained 0.4 kg. She went on to complete recovery, as indicated in Table X, although there was occasional chemical evidence of low plasma potassium later in the experiment. This represents the only case and type of chronic toxicity encountered, and suggests that at high doses and low food intake, Diamox may threaten potassium balance in the dog. The sequence observed suggests that the signs of depression and anorexia may be due to potassium depletion. In our long-term studies of electrolyte excretion in the dog there have been noted occasional days where there was some net potassium loss. This is distinctly the exception to the more general observation that the measured urinary components, taken on a net 24-hour basis, return to normal after the first few days of Diamox (15).

In the two highest dose dogs (P27 and M73) there was slight anemia at some occasions during treatment. In dog M73, which had a final hemoglobin value of 7.2 mM. O_2 cap./l. at the end of treatment, there was reversion to normal (10.0) values when drug was stopped. The dogs on 33 mg./kg. and 10 mg./kg. never showed anemia. None of the animals showed any significant change in total or differential white cell counts.

Transient periods of weight loss on the 33 mg./kg. and 10 mg./kg. dose were correlated with alterations in the feeding schedule at times where electrolytes were being studied. Alterations such as a shift of feeding time from 1 P.M. to 9 A.M. occasionally resulted in several weeks of diminished intake.

TABLE X

Long-Term Toxicity and Electrolyte Data in Adult Dogs Receiving High Doses of Diamox

Dog No. and Sex	Drug Administration		Body Wt.		Hematology						Excretion of Drug, % Dose in 24 Hr. Urine		Plasma Electrolytes (24 hrs. after dose)						Fasting Blood Glucose
	Dose	Oral Period, Days*	Day*	Kg.	Day*	RBC†	Hgb‡	WBC§	Neut¶	Lym¶	Day*	%	Day*	pH	Meq./l.				
															HCO_3^-	Na^+	K^+	Cl^-	
	mg/kg/day																		mg.%
P27 female	100	1–140	0	10.9	0	7.0	11.3	10.8	74	18	1	19	0	7.35	20	144	4.0	109	100
													3	7.27	17	133	—	103	
			15	8.7	18	6.4	8.5	11.1	81	15	3	92	17	7.16	12	123	3.2	104	136
			32	8.5	45	6.8	8.6	14.3	74	20	8	64	45	7.13	14	137	3.0	114	108
			56	9.6	86		8.5				17	44	86	7.15	14	135	3.2	118	111
			85	10.0	100		9.1				140	51	141	7.19	16	140	3.3	113	
	0	141–147	147	11.0	128		9.2				148	27	148	7.37	20	132	3.6	102	
	100	148–478	214	8.4							163	29	149	7.22	14	134	3.0	109	
			242	7.4	246		8.2	13.7	75	16			226	7.11	15	132	3.0	113	117
			280	10.2									280	7.14	15	138	2.4	118	
			415	9.6‖									427	7.22	16	144	2.6	110	
			455	8.7‖									458	7.15	15	142	3.3	110	
	Last dose, sacrificed	478	476	9.5	476	5.3	7.3	14.8	61	21									
M73 female	100	1–140	0	11.2	0	6.5	9.5	9.9	63	24	1	27	0	7.32	20	140	4.4	109	98
													3	7.32	18	139	—	111	
			15	9.5	18	7.0	8.9	10.8	87	8	3	87	17	7.16	18	142	3.4	106	127
			37	8.9	45	6.1	7.8	15.4	68	15	8	62	45	7.12	12	146	3.7	117	99
			42	10.0	87	5.9	5.4	22.1	81	11	17	140	86	7.14	13	131	3.2	112	122
			99	12.0	100		7.2				140	14	141	7.21	16	142	3.3	115	
	0	141–147	123	9.7	128		6.8				148	18	148	7.36	21	140	3.5	105	
	100	148–477	147	9.6							163	58	149	7.27	15	135	3.4	109	
			225	11.3	246		9.4	17.1	71	13			226	7.26	16	137	3.7	112	101
			280	14.5									330	7.17	16	132	4.0	111	
			360	13.6									427	7.27	18	145	2.9	107	
			455	9.9‖									477	7.10	14	145	2.5	121	
	Last dose, not sacrificed	477	476	11.4	476	5.3	7.2	17.9	68	10			479	7.24	17	146	3.5	111	
													481	7.38	24	147	3.4	106	

M78 female	33	1–52	0	11.0	0		8.0				58	53	0	7.34	19	144	3.7	104	94
	0	53–57	54	12.1									59	7.25		140	3.4		
	33	58–361	82	11.9									64	7.25		134	3.0		
			110	11.0	128		10.0	14.8	56	20	162	49	107	7.28	15	134	3.7	119	103
			162	14.4									161	7.20	17	134	3.4	121	
			283	14.4									309	7.29	18	142	3.0	113	
			358	11.1‖	358	6.8	9.9	13.0	51	30			338	7.13	13	144	3.3	115	
	Last dose, sacrificed	361																	
P21 female	33	1–52	0	11.1	0		7.8				58	27	0	7.30	20	140	3.8	100	104
	0	53–57	54	12.7									59	7.21		145	3.4		
	33	58–62	82	12.3									60	7.16		142	3.1		
	0	63–70	110	12.4									67	7.31		141	3.6		
	33	71–365	162	14.5									80	7.07	16	142	3.4	117	
													107	7.24	21	132	3.3	108	129
			282	13.4	128		9.3	14.1	70	17	162	35	162	7.20	18	132	3.3	121	
			358	12.6‖	358	7.1	8.8	17.0	67	14			340	7.13	14	138	3.2	110	
	Last dose, sacrificed	365																	
A42 female			0	14.8							1	73	0	7.30	24	139	3.6		
	10	1–7	10	14.4									3	7.25	19	138	4.0		
	0	8–13	72	15.4									11	7.30	20	136	4.0		
	10	14–245	132	17.2									246	7.20	20	152	4.1	115	
	0	246	190	17.3									247	7.29	22	152	4.1	122	
	10	247–274	251	17.0‖									275	7.33	20	144	3.6	105	
	0	275–281	272	18.3	272	5.9	8.2	15.7	74	12			276	7.38	23	146	4.0		
	Sacrificed	281																	

* Days of experiment: 160 is, for example, the 160th day of experiment, not necessarily the 160th dose.
† Millions per mm³.
‡ mM O_2 capacity/liter.
§ Thousands per mm³.
¶ Per hundred white cells.
‖ Body weight loss associated with decreased food intake concident with change in feeding schedule during electrolyte studies.

Supplement to Table X: General Behavior of Dogs

Dog P27 Day 1–2: Behavior normal. Day 3–37: Vomiting, listless, poor appetite, respiration deep, muscular tremor, unsteady gait. Day 38–213: Normal appetite, condition improving. Behavior usually normal except for listlessness which accompanies periods of decreased food intake. Day 214–258: Poor appetite. Day 259–407: Normal appetite and behavior. Day 408–457: Poor appetite and weight loss, at time of special experiments where feeding time was altered. Day 458–478: Normal appetite.

Dog M73 Day 1–2: Behavior normal. Day 3–15: Vomiting, listless, poor appetite, respiration deep, muscular tremor. Day 16–29: Fair appetite, behavior improving. Day 30–37: No appetite. Same symptoms as on days 3–15. Day 38–97: Normal appetite. Day 38–104: Pregnant. Delivery of 2 puppies, well-formed but ½ normal size, both died a few days after birth. Day 105–140: Successfully nursed 2 foster puppies. Unusually nervous and excitable during this time. Day 98–130: Poor appetite. Day 131–405: Normal appetite. Day 406–457: as for P27 above. Day 458–477: Normal appetite. Day 264 and 442: in heat.

Dog M78 General behavior normal throughout test. Day 158: in heat.

Dog P21 General behavior normal throughout test.

Dog A42 General behavior normal throughout test.

These five dogs were followed clinically and chemically (15) for 16 months in the case of the higher dose and 12 months for the lower doses. The only exception to their general well-being throughout was noted above. Four of the five dogs (M73 being excluded) were sacrificed from several hours to 6 days after their last dose (Table X). The positive pathological findings follow.

(2) *Post-Mortem Studies.* The post-mortem findings in the four sacrificed dogs which received Diamox for 9–16 months are listed in Table XI. The nutritional state of the dogs is not given in the table and may be described as follows: The dog which had received 100 mg./kg./day for 16 months (P27) was and remained lean, while the two dogs which received 33 mg./kg./day for 12 months (P21 and M78) had a moderate amount of fat. The dog which received 10 mg./kg./day for 9 months (A42) was and remained obese.

Few findings of Table XI need discussion. The pigment in the liver of the dogs was iron and sudan negative, but its nature could not be determined by various histochemical methods. The brown material in the lungs of A42 was possibly "formalin pigment". The histologic diagnosis of calcium deposits along the alveolar walls of dog P27 was confirmed chemically. The calcium content of the lungs of this dog was 19 g./kg. dry lung, while that of dog M78, which had no visible calcium deposits in the lungs, was 0.55 g./kg. dry lung.[6] Similar calcium precipitates were found in the 4-month old puppy P54 which had received 20 mg./kg./day for 4 months. [See below, Section III, B, 2, b (2).]

[6] We wish to thank Dr. P. R. Averell, Dr. S. B. Davis and Mr. R. P. Smith of these laboratories for the calcium analyses, various phases of which were carried out under their direction.

TABLE XI

Summary of Scope and Results of Post-mortem Studies in Four Adult Female Dogs Which Received Diamox for 9 to 16 Months

Dog No.	Dose	Age at Death	Dur. Drug Adm.	Morph. Proced.	Circ. Syst.	Resp. Syst.	Digest. Tube	Liv.	Panc.	Kidn.	Uret. Blad.	Femor. Marr.	Spleen	Thyr. Parath.	Pituit.	Adren.	Repr. Syst.	Centr. Nerv.	Eye
	mg/kg																		
A42	10	6 y	9 m	Ma.	+	+	+	+	+	+	+	+	+	+		+	+	+	
				Mi.		p		v		+				+		+		p	
P21	33	4⅛ y	12 m	Ma.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
				Mi.		+		v		v				+		+		p	
M78	33	3 y	12 m	Ma.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
				Mi.		+		v	+	+		+		+		+		+	
P27	100	3 y	16 m	Ma.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
				Mi.		p		v	+	+		+		+		+			

Explanation of Abbreviations: Under Age at Death and Duration of Drug Administration: y = year, m = months, d = days. Under Morphologic Procedures: Ma. = macroscopic, Mi. = microscopic. Under Organs: + = normal, v = non-pathologic variation, p = pathologic, blank = not examined.

Explanation of non-pathologic variations and pathologic findings:

A42: Respirat. System: Alveolar edema in some areas. Brown pigment (besides carbon) in pulmonary connective tissue. Liver: Brown pigment in endothelial cells. Fat storage in scattered cord cells (unusual type). Centr. Nerv. System: Small bodies of unknown nature in the brain substance.

P21: Liver: Brown pigment in endothelial cells. Kidney: Lymphocytic masses in pelvis. Centr. Nerv. System: Small bodies of unknown nature in the brain substance (possibly parasitic).

M78: Liver: Brown pigment in endothelial cells.

P27: Respirat. System: Calcium deposits along alveolar walls. Liver: Brown pigment in endothelial cells.

It was found that the dog colony of these laboratories, and particularly P27, inadvertently received high doses of vitamin D prior to and during part of this work. P27 received 3600 units vitamin D/kg. daily for 22 days in August 1950 when she was 4 months old. This appears from our records to have been given by error. Thereafter she received 100–200 units/kg./day, the standard dose in the colony for adult dogs, until January 1953. Then, due to circumstances unrelated to the present work, this was increased in the colony to about 700–800 units/kg./day. The present work was done in 1952–1953. P27 was sacrificed in August 1953. The puppy P54, along with the rest of his litter (Fig. 7), received 560–900 units/kg./day for 1 month after weaning; thereafter 240–560 units/kg. until sacrifice 3 months after weaning. Optimal vitamin D requirement in dogs is reported to be 72 units/kg. (48). Calcium intake in this colony is 1–2 g./day/dog. In adults this is virtually all in the dog chow; in puppies this is a total of the amount in chow plus added daily calcium acid phosphate.

The calcium deposits in the lungs can probably be ascribed to overdoses of vitamin D. Hendricks *et al.* (48) have shown that after excess vitamin D is given to dogs, calcium is strikingly elevated and apparently fixed in the lungs, for its concentration in lung shows no decline 5 months after the end of treatment. In this time, other organs (kidney, heart, muscle) showed marked decrease in calcium content, in many cases to normal. In their study scars were seen in the places of former calcification. There were no scars in the kidneys of P27 and P54, but the lungs of these dogs appear morphologically similar to those described following hypervitaminosis D (48, 49, 50).

Data on calcium and phosphorus metabolism in relation to Diamox are discussed below (Section IV, E).

(3) *Interpretations.* The morphological findings are interpreted as showing no serious damage to any organ or physiological system, and agree in this respect with the observations during life. Thus it appears that dogs can receive oral treatment with Diamox for prolonged periods, considerably above the therapeutic range of dosage (see Discussion), with no serious or irreversible effects.

In addition to the five dogs in which the detailed data of Table X were obtained, eight other dogs were treated in the course of electrolyte studies (15). They received doses of 5 mg./kg./day, 10 mg./kg./day, and 10 mg./kg./12 hours. Such schedules were followed for various times, ranging from 2 weeks to 9 months. No unusual phenomena were noted during life. One dog (M14) on 10 mg./kg./12 hours for 2 months was also autopsied.

Prior to these studies, in 1951, M14 underwent removal of multiple benign tumors of the mammary glands. The post-mortem study of this dog revealed multiple, so-called benign, tumors of the endocrine glands; a large one in the right adrenal, a small one in the left adrenal, a small one in the left thyroid and a cystic type in the anterior and intermediary part of the hypophysis. Besides these findings there were old-age changes in the kidneys but no other significant lesions. This dog was 8 years of age; others in this study were not above 6 years. Interpretation of these findings in relation to Diamox is beyond the scope of this paper.

b. Growth Studies in Young Rats and Dogs. (1) *Drug-Diet in Rats.* Twenty-five males and twenty-five females were received at 4 weeks of age from Carworth Farms. After an observation period of 2 weeks animals of each sex

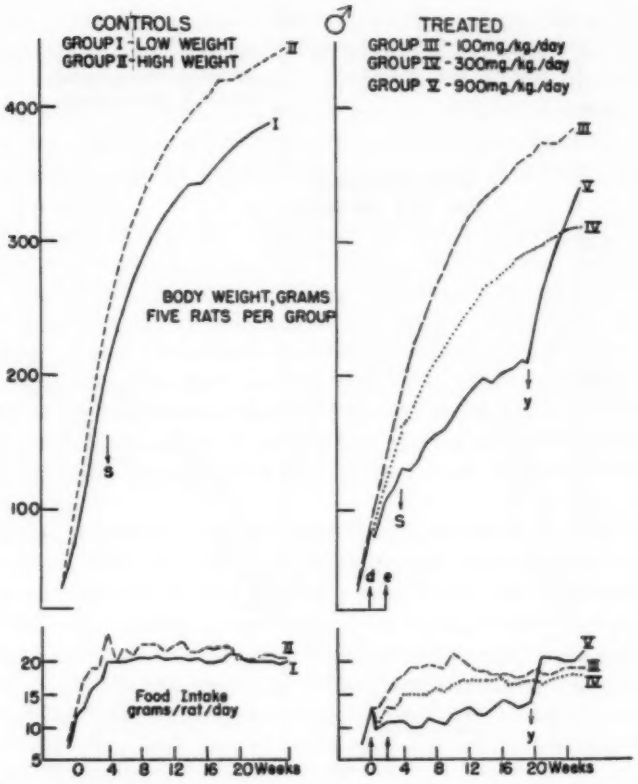

FIG. 5a. Young male rats receiving Diamox in diet. Body weight and food intake. Five animals per group.

Explanation of Symbols in Figure

d. Starting dose: Group III, 50 mg./kg./day; Group IV, 150 mg./kg./day; Group V, 300 mg./kg./day.
e. Doses increased after 4 weeks; Group III, 100 mg./kg./day; Group IV, 300 mg./kg./day; Group V, 900 mg./kg./day
s. One animal each from Groups I (control) and V (highest dose) sacrificed. See text.
y. Drug discontinued in Group V. See text.

were placed in five groups based on minimal variation in body weight. The groups of lowest and highest weight were reserved as untreated controls. The three intermediate groups of each sex were assigned drug doses, as indicated in Figure 5. In the subsequent 6 months these animals were studied with respect to pharmacology (Tables V and IX), electrolytes (15), body weight and food intake (Fig. 5), hematology and pathology.

All animals appeared well and normally active throughout the test. It is of

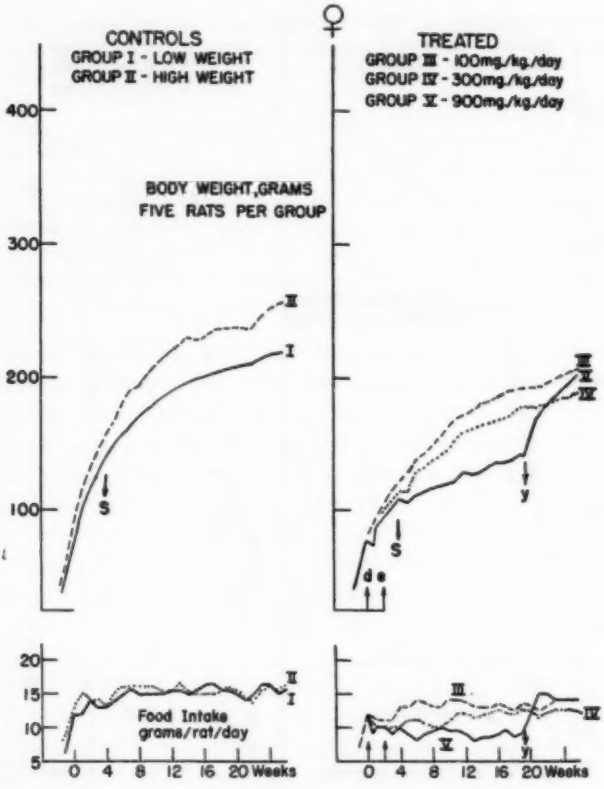

FIG. 5b. Young female rats receiving Diamox in diet. Body weight and food intake. Five rats per group.

For explanation of symbols, etc., see legend for Figure 5a.

Standard deviations of weights in all groups of rats (Figs. 5a and 5b) are as follows (See also section III, B, 2b for system of arrangement of groups):

Group	Grams				Grams			
	Sex	Initial	Weeks		Sex	Initial	Weeks	
			20	26			20	26
I–II	M	10.9	31.5	32.6	F	7.2	23.3	26.1
III	M	2.6	26.1	25.6	F	1.4	8.3	14.8
IV	M	2.5	19.3	22.1	F	1.2	19.2	14.0
V	M	2.9	28.8	37.6	F	2.0	24.4	12.9

interest that on the highest doses there was a striking metabolic acidosis (15) but no signs clearly referable to this. Figure 5 shows that at 100 mg./kg./day, weight gain was almost equal to that of controls. In addition, we have measured the skeletal lengths of these rats, which were no different from the control group. On 300 mg./kg./day, weight gain was somewhat inhibited; skeletal length was normal. On the highest (900 mg./kg./day) dose both weight and length were retarded.

Since the deficiency in weight and length could be correlated with decreased food intake, it was of interest to find whether the rats would gain when returned to normal diet. At point y in Figure 5, animals on the highest dose were taken off drug, whereupon food intake and weight rose sharply to control values. After 6 weeks of normal food intake, skeletal length also reached normal values. It seems clear that the slowing of weight gain and skeletal growth was reversible. Whether the inhibition was due to the drug or to lowered food intake alone (38) cannot be decided from these data. It is significant that such inhibition was demonstrated only at doses over 30 times those of the therapeutic range.

At point s in Figure 5, a time of rapid growth, an animal of each sex from the control and from the highest dose groups was sacrificed and tissues examined grossly and under the dissecting microscope. The lower weights of animals on the higher doses seemed largely due to the relative paucity of fatty tissue. No gross abnormalities were found. Epiphyseal development in the long bones was studied by examining cut-surfaces under the dissecting microscope: stages were the same in control and treated rats. Histological examination of thyroids and testes showed no differences between control and treated rats; testes were mature in both.

Hematological studies were carried out on twelve drug-treated and eight control animals on two occasions, the 14th and 25th week. Eleven drug-treated rats showed a normal blood picture. The remaining animal (No. 2), from the highest dose group, exhibited a bizarre pattern, characterized by an anemia with increase in nucleated red cells, and the appearance of unclassifiable ameboid cells. When these cells formed 88% of the supposed white count, the number of "white cells" per cubic millimeter was 100,000 (10–20,000 is normal); the numbers of neutrophil leukocytes and lymphocytes per cubic millimeter were normal at all times. This rat was not sacrificed at the appointed time, but taken off drug for 6 weeks, during which time the blood picture became normal. On resumption of drug, the ameboid cells reappeared, and by the 12th week of treatment accounted for 63% of total "white cells." Anemia, similar to that noted above, also reappeared. After 19 weeks (or a total of 45 weeks of drug) the rat was finally sacrificed. There were large numbers of normoblasts in femoral marrow and spleen, and large quantities of iron pigment in spleen, confirming the diagnosis of hemolytic anemia fairly well compensated by high erythropoietic regeneration. The nature of the unclassifiable cells was not clarified by histological studies. It remains, of course, an open question whether or not the appearance of these peculiar cells interfered with any known function. Leukopenia, agranulocytosis or other functionally significant disturbances of the leucopoietic apparatus did not occur in any of the drug-treated rats.

During the course of the experiment of Figure 5, two rats, females of Group IV, died. Autopsies failed to show any significant macroscopic lesions. Since all other animals survived this and three times this dose, there is no indication that these deaths were related to drug treatment.

Of the 50 animals, seven are discussed above; the remaining 43 were sacrificed 6 months after the onset of treatment and studied as follows:

1. For chemical studies, killed by bleeding from the heart.
   a. Used for Blood Electrolytes (15) 17
   b. Used for analysis of Diamox in tissues (Table IX) 4
2. For morphological studies, killed by intraperitoneal pentobarbital. Results below.
   a. Used for complete autopsies 10
   b. Used for abbreviated autopsies 12

Morphological Findings in Rats. There were no pathologic macroscopic findings. No microscopic lesions were found in rats on 100 mg./kg./day. One rat on 300 mg./kg./day showed bile droplets in some renal tubular cells. One rat on 900 mg./kg./day showed extensive casts (probably calcium) and some indication of healed injury in the kidneys. The same animal, and one other, showed an unusual amount and distribution of visible lipids in the liver.

These observations are interpreted as showing that at some time there was transient and minor liver injury in three of the rats; there was no necrosis or scarring. The renal casts seen in the highest dose rat are reminiscent of lung findings in adult dog P27 cited above. It is felt that these casts probably contain calcium. However, there has been no other evidence to incriminate Diamox in causing alteration in calcium metabolism, either from studies of plasma calcium and phosphate in adult dogs which received Diamox for 16 months (Table XII) or from our work and that of others on the effect of Diamox on excretion of these ions [see Section IV, E and (15)]. X-ray findings in the following section are also relevant.

(2) *Oral Administration in Dogs.* The following experiments were performed to judge the effects of Diamox in growing dogs, including animals in which the drug was used from the day of birth. This latter program is not one for which any therapeutic parallel in man was foreseen, but rather an attempt to find whether carbonic anhydrase inhibition is injurious to the new-born animal.

The puppies were from a single litter, and were divided at birth into three groups. Group (a) were untreated controls nursed by their own mother until they were weaned at 5 weeks of age, and then continued under normal laboratory regimen. Group (b) were similarly nursed and weaned, and on weaning were started on 20 mg./kg./day of Diamox by capsule. The arrow at 5 weeks in Figure 6 indicates the onset of oral dosage. Group (c) received Diamox from birth in the milk of dog M73, who nursed this group while on 100 mg./kg./day. At weaning, they, like Group (b), received 20 mg./kg./day by cap-

TABLE XII

*Plasma Calcium and Phosphorus Concentrations in Long-Term Treatment with Diamox**

Dog No. and Sex	Dose, oral	Day*	Plasma†	
			Ca^{++}	P
	mg./kg./day		*meq./l.*	*mM/l.*
P27, female	100	87	5.0	
		107	5.1	
		427	4.9	1.6
		458	4.9	1.1
M73, female	100	87	4.8	
		427	5.5	1.8
		457	5.0	1.4
M78, female	33	309	5.0	1.3
		337		1.4
		338	5.2	
P21, female	33	332	5.1	1.2
		339	5.1	1.5
A42, female	10	233	4.7	1.2
		238	5.6	1.3
		247	5.8	1.6
		248	5.6	

* Full schedule in Table X. Day is that of experiment, not necessarily of dosage.

† Control plasma Ca^{++} values in this laboratory ranged between 5.1 and 5.6 meq./l. (8 determinations). Control P values in this laboratory ranged between 1.2 and 1.6 mM/l. (5 determinations).

sule. As noted above in Section II, C, the milk of dog M73 contained considerable Diamox, and the puppies had detectable levels of the drug in their blood, presumably from the onset of nursing.

Figure 6 shows that the growth of treated and control dogs was essentially the same, particularly when judged by rate of gain. The treated dogs were indistinguishable from their controls in activity and appetite. X-rays indicated no difference in bone development among any of the seven dogs. Electrolyte studies at 3 and 4 months showed that the treated animals had a slight acidosis, 24 hours after the previous dose of Diamox. Average figures were pH 7.28, and HCO_3^- 19.2 meq./l. Sodium, potassium and chloride concentrations in plasma were normal. Hematological findings were unremarkable. Blood glucose was normal 2 to 4 hours after feeding.

At 4 months these dogs, from an unusual outside contact, contracted distemper. At the time of this incident they had not yet been vaccinated. They were given active and passive immunization at the onset of illness. The three control dogs had convulsions; one died in such an attack and the other two were finally sacrificed. One of the four treated dogs (P54) convulsed; the other three showed mild respiratory and intestinal symptoms. These dogs were also sacrificed, and complete autopsies performed. This accidental experiments suggests that Diamox does not seriously alter the progress of this disease.

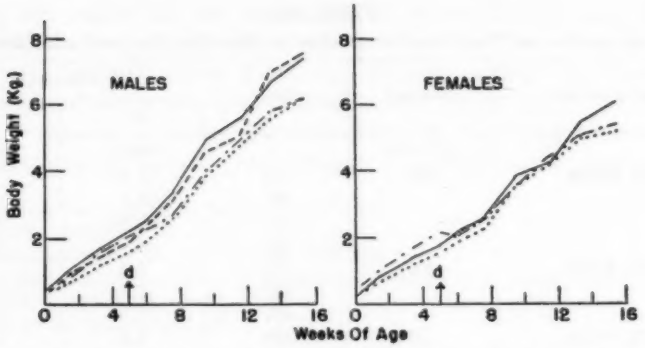

FIG. 6. Body weights of puppies on Diamox from birth and from weaning. Arrow at d: day of weaning.

(a) Untreated controls: ——— male M52, and female M55; ---- male M53.

(b) 20 mg./kg. from weaning: ----- male M51, and female M57.

(c) Nursed from birth by foster mother treated with Diamox (M73, 100 mg./kg./day) then 20 mg./kg. from weaning: ····· male M54 and female M56.

Morphological Findings in Growing Dogs. Skeletal development and reproductive organs were normal for the ages of these dogs. All organs examined were free from pathological change with the exception of the lungs of P54. They showed areas of conspicuous emphysema, with many microscopic deposits of hematoxylin staining material in the pulmonary tissue. This finding is similar to that described and discussed above in one of the long-term chronic toxicity dogs (see P27 above and Section IV, E below).

These experiments on the growing rat and dog appear to show conclusively that Diamox, in doses considerably above the therapeutic range, has no adverse effect on growth and development in these species.

IV. SPECIAL STUDIES

In addition to the general pharmacological studies described above, certain exploratory experiments or observations were made which relate more directly to possible toxicity deriving from the specific role of Diamox as a carbonic anhydrase inhibitor. The need for such work scarcely requires emphasis, since Diamox is the first carbonic anhydrase inhibitor of therapeutic promise, and since the extensive role of this enzyme in mammalian physiology is evident (Table II). More studies will be needed; as indicated, some are in progress here as well as in other laboratories.

A. Effect of Diamox on Red Cell Carbonic Anhydrase

Because of the high concentration of carbonic anhydrase in red blood cells (1) and in view of the finding that Diamox was concentrated and persisted

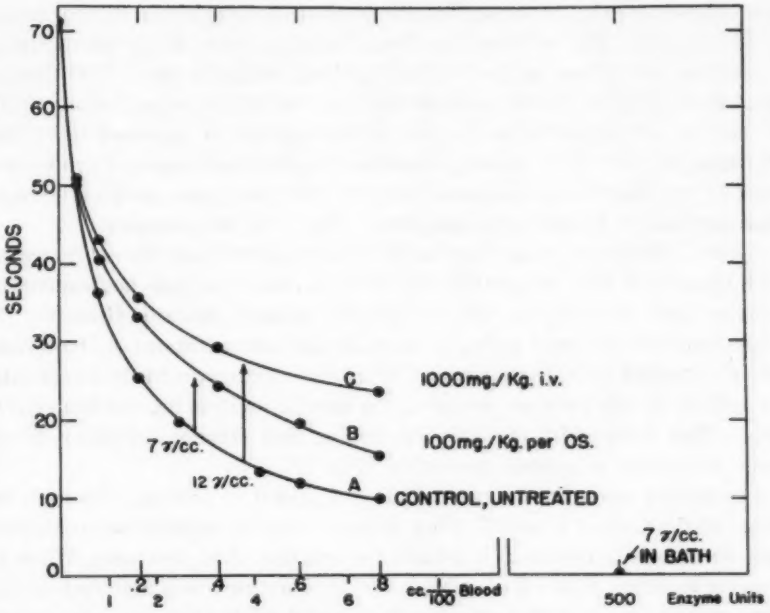

FIG. 7. Enzyme activity of blood of dogs treated with Diamox.

Determinations done by the method described in the accompanying paper (30). Enzyme for the 500 unit point was purified material according to the method of Mann and Keilin (4).

Curve A. Normal dog blood. Untreated.

Curve B. Activity of whole blood from two dogs (P27 and M73) which received 100 mg./kg. by mouth 24 hours previously. Averages are plotted; individual points did not vary more than 1 second between blood from the two dogs.

Curve C. Average plasma concentration of Diamox was 2.0 γ/ml.; R.B.C. concentration of Diamox was 14 γ/ml. Similar to Curve B but from two dogs (P35 and P36), which received 1000 mg./kg. intravenously 24 hours previously, average plasma concentration of Diamox was 11 γ/ml.; R.B.C. concentration of Diamox was 14 γ/ml.

Curves B and C are displaced upward from Curve A. But when the same amount of Diamox present in blood B or C was added to blood A (and equilibrated), A was displaced upward to the extent indicated by the arrows, i.e. blood A then fell along Curves B or C. Thus, the shape and position of Curves B and C are exclusively due to Diamox itself.

Extrapolation of Curves B and C downward (to the right) suggests than as enzyme and inhibitor are increased together, which would approach the situation in whole blood, the reaction becomes faster. The extreme point on the right of the abscissa indicates that when enzyme and drug are present in the bath in amounts approaching their actual concentration in blood, the reaction is instantaneous. See text.

in erythrocytes (Fig. 3), we studied the effect of the drug on the enzyme system in this location. The principal handicap in such a study is the concentration of carbonic anhydrase in the circulating blood, which is about 1000 times as great as used in the *in vitro* system which served for the present studies (30); if the enzyme concentration in this *in vitro* system is increased more than 10 times, the rate of the reaction becomes too fast to measure. For measurement of very high concentrations of enzyme new techniques, such as the rapid-flow methods of Chance and Roughton (32a), will be necessary.

Figure 7 shows enzyme activity of blood from controls and from dogs treated with Diamox. It may be seen that the blood of treated animals has less enzyme activity than controls, i.e., the curves are displaced upward. However, this displacement is the same as found when similar concentrations of Diamox are added to control blood (note arrows). Thus, the effect on the blood is accounted for entirely by the Diamox present at the time; inhibition has not been cumulative. This agrees with our previous finding that Diamox inhibition of carbonic anhydrase is entirely reversible (Fig. 2).

The second, and perhaps more significant, point of this experiment is the shape of the Curves B and C. They indicate that as more blood (containing enzyme and drug together) is added, the reaction time decreases. When increasing amounts of blood are added, the situation *in vivo* is approached; it is clear that far short of the situation for true blood the reaction will become instantaneous. The point on the extreme right on the abscissa represents 500 enzyme units of purified material, or 71 units/cc. in the bath. This is only about 10% of the enzyme concentration of blood; nevertheless, Diamox in the bath at a typical blood concentration does not alter the instantaneous reaction time.

These experiments suggest that Diamox in higher than therapeutic doses does not radically change the carbonic anhydrase activity of the blood. The reason for this seems simply to be the huge concentration of enzyme in the blood. More delicate methods may detect small effects of Diamox directly on carbonic anhydrase in blood, since it is possible in certain circumstances (15, 20, 20a) to show an elevation of plasma pCO_2 after Diamox. This may reasonably be ascribed to interference with CO_2 elimination by inhibition of blood enzyme and subsequent slowing of the dehydration of carbonic acid within the erythrocyte.

B. Blood Glucose, and Insulin Tolerance

Since it has been reported that acidosis may interfere with the action of insulin (39), it was of interest to observe the chronic acidotic dogs (Table X) in this connection. In the dogs maintained on 100 mg./kg./day (M73 and P27) there was a slight but definite tendency for the fasting blood glucose to

rise in the first few months of treatment (Table X). Glucose tolerance tests were not done.

A single insulin tolerance test was performed on P27, 7 weeks after the onset of treatment. Blood pH was 7.17. A dose of insulin (0.2 unit/kg.) was given and blood sugar levels (Folin-Malmros, mg.%) were as follows, with those of an untreated dog done simultaneously noted in parentheses: Fasting, 112 (90); 15 minutes, 68 (64); 30 minutes, 76 (61); 45 minutes, 71 (65); 1 hour, 78 (83); 2 hours, 111 (115); 3 hours, 127 (111). It seemed clear that the two responses were not substantially different, and that insulin tolerance was normal in the dog treated with Diamox.

The acidosis studied here was less severe than that induced by ammonium chloride in the earlier study of insulin resistance (39), and also differed in being long-term rather than of a few days' duration. Also, as will be discussed elsewhere (15), the nature of the acidosis following Diamox is somewhat different from that of ammonium chloride, although both are fundamentally "metabolic" in type.

C. Effect on Renal Filtration and Secretion

As indicated in Figure 3c, creatinine clearance in the dog was slightly and transiently reduced by intravenous Diamox (5 mg./kg.). The increase in urinary pH far outlasted the small decrease in clearance. Berliner (14) noted some reduction in inulin clearance in the dog after higher intravenous doses and under somewhat different conditions. Large *oral* doses of Diamox, however, do not alter creatinine clearance in the dog, either in the initial period of diuresis or during recovery from that diuresis (15).

Creatinine clearance appears normal or somewhat elevated during the long-term acidosis recorded in Table X. Such clearances were determined in two dogs. The values in ml./min. were as follows:

Dog M78 control clearance, 1 month before start of drug: 36–46, average 42. After 80 days of drug administration, 4 hours after dose: 47–58, average 51.

Dog P21 control clearance, 1 month before start of drug: 26–31, average 29. After 80 days of drug administration, 4 hours after dose: 33–57, average 41.

The effect of Diamox on renal secretion of phenol red was studied in two dogs. Six mg. of phenol red was given intravenously. Thirty minutes later Diamox was given intravenously, in one case 2 mg./kg. and the other 50 mg./kg. At the end of 2 hours, 90 and 73%, respectively, had been excreted (see Fig. 3d). This is well within normal limits. In both cases typical alkalinization of the urine was observed. This suggests that the carbonic anhydrase system in the kidney is independent of that responsible for the secretion of phenol red.

D. Effect of Diamox in Pregnancy

One of the highest dose (100 mg./kg./day) chronic toxicity dogs (M73, Table X) had delivered a normal litter of five, 5 months prior to onset of Diamox. Five weeks after the onset of drug treatment she was mated and went through a normal term of pregnancy. She delivered two undersized

puppies which did not survive. One puppy died one day after birth, of subdural and intraventricular hemorrhage. The second died 5 days after birth; at autopsy there was evidence of pneumonia with venous congestion of liver and lungs. There was an area of necrosis at the apex of the myocardium.

The mother (M73) had undergone a period (see Section III, B, 2a) of anorexia, weight loss and general debility at the onset of drug treatment, just prior to mating. Six months after M73 was taken off Diamox, she was mated again and delivered a litter of five normal puppies, still normal at the present age of 4 months. This suggests reversibility of those mechanisms which were responsible for abnormalities in the litter born while on Diamox.

This incident, although it occurred at a dose 10–20 times the human therapeutic dose, indicated the need for further studies, particularly since Diamox seemed worthy of clinical trial in edema due to the toxemias of pregnancy. A replica of the M73 experiment has not yet been carried out. The following observations, however, are pertinent to the effect of Diamox in pregnancy.

A second dog (P20) received 33 mg./kg./day orally for the last third of gestation and 5 days post-partum. At term she delivered a litter of five, all of which developed normally and at 6 weeks weighed 1.7–2.8 kg., which is average for this laboratory.

Thirteen pregnant rats received Diamox in the diet for the last week of term at doses from 50–300 mg./kg./day, together with three untreated controls which had been mated at the same time. All sixteen animals appeared to have normal parturition. The thirteen experimental litters ranged from 3 to 13 totalling 107 young, while the three control litters ranged from 6 to 12, with a total of twenty-four young. All new-born rats were followed to 5 weeks of age. At the end of this period 102 experimental young rats and 18 controls survived.

In subsequent studies by Thiersch (40) 100 mg./kg./day of Diamox was given orally to rats from the 4–15th day of pregnancy, and in a second group from the 8–17th day. Assuming average implantation at 5–6 days, the periods prior to, during and following fetal implantation in the uterus were covered. There was no interference either with fetal or maternal development.

Work of a preliminary nature was also done in rabbits and guinea pigs (41). Large single doses of Diamox were given at several critical points in the gestation period, starting 24 hours after copulation, without any injury to fetuses or mother.

These studies indicate the following effect of Diamox on fetal development or parturition: except for the single experience in one dog (M73) under extreme conditions of time and dose, no deleterious effects were observed in a variety of species and conditions.

The maternal post-partum period was without incident in the two dogs and thirteen rats studied here. It may be recalled that in spite of the high daily

doses given to Dog M73, she had normal parturition and was able to foster-nurse two of the litter (c) charted in Figure 6.

E. Effect on Calcium and Phosphorus Metabolism

Because of the association of "renal acidosis" with decalcification emphasized by Albright *et al.* (42), the possible effect of Diamox on calcium and phosphate values, and on growing and adult bones was investigated. The defect in renal acidosis in children (45) and in adults [Milkman's Syndrome (42)] is superficially similar to the (reversible) situation in high dose treatment with Diamox (15), i.e., metabolic acidosis, with failure to excrete acid and ammonium maximally. However, we have shown that in prolonged acidosis following Diamox, daily urinary $NH_4^+ + H^+$ is *normal* (15), whereas in Milkman's Syndrome it is *subnormal* (42). This difference may be critical; in Milkman's Syndrome it is postulated that calcium is mobilized as a base (Na^+ and K^+) sparer when normal urinary acidification fails. The result is nephrocalcinosis and osteomalacia; chemically there is low or normal plasma calcium and low plasma phosphate. As will be indicated in the next paragraph, calcium does *not* appear to be mobilized in acidosis due to Diamox, which may be a reflection of the *normal* net daily urinary composition in this situation.

The following findings appear to show that under several different sets of conditions Diamox has no appreciable effect on calcium or phosphate metabolism. Initial or continual doses of Diamox do not affect phosphate excretion in the dog (15) or in man (18). In man, Diamox did not increase urinary calcium excretion over an 8-day treatment period (43). In the rat, 50 mg./kg./day (which induced acidosis) for 16 days gave no evidence of decalcification, as shown by direct measurements of bone ash (58). In the five long-term dogs of Table X, plasma Ca^{++} and inorganic P determinations were done and recorded in Table XII. They appear entirely normal. Bones of the rats and dogs used in the above growth and toxicity studies were examined at autopsy, and found normal. X-rays of the skeletons of the growing litter (Fig. 6) showed no difference between treated and control animals. Although calcium has been found in the lungs of two dogs (see above) receiving Diamox, neither of these nor any other dogs in our series showed *renal* calcification or residues of calcification. Two rats on the 900 mg./kg./day dose (100–200 times the therapeutic dose) did show microscopic evidence of calcium in renal tubules. It appears important to make the distinction between persistent *lung* calcium probably due to retention following hypervitaminosis D, and *renal* calcium which may be due either to hypervitaminosis D (excess calcium) or mobilization of calcium from bones (in calcium depletion).

Lastly, we cannot exclude an interaction between the potential calcium-retaining action of vitamin D and high calcium intake in our dogs, and the possible loss of calcium in acidosis, as suggested by Albright. The experiments of Jaffe *et al.* (57) show clearly that high calcium intake can ameliorate the decalcification which follows NH_4Cl acidosis in dogs. It is conceivable that in our dogs such interactions prevented calcium loss from bone in acidosis induced by Diamox. (Although, as indicated above, there are at present no theoretical or factual data suggesting that Diamox actually alters calcium balance.) If indications of calcium loss on Diamox ever should appear, treatment with vitamin D and calcium could be expected to overcome it.

F. Effect on the Crystalline Lens

Organs rich in carbonic anhydrase merit particular attention after Diamox treatment. Kidney, pancreas, and gastric mucosa have been examined in connection with the autopsies cited above, and found normal in long-term treated animals. The lens, however, actually contains more carbonic anhydrase than any other tissue except the erythrocyte (17). We have not attempted a morphological post-mortem study of the lenses of animals treated with Diamox. During life, conjunctivae, cornea, and pupillary reflexes were found normal in dogs receiving Diamox. Five months after the cessation of treatment of M73 (100 mg./kg./day for 16 months), she was given an ophthalmological examination by Dr. E. J. Ballintine, who has kindly given permission to quote the following: "Examination of the eyes with ophthalmoscope and with good illumination reveals no abnormalities of lens, cornea or fundus." Since this represents a very long exposure to Diamox at 10–20 times the therapeutic dose, it appeared that in this dog Diamox did not produce visible injury to the lens.

In the two rabbit experiments of Table VIII, concentration of Diamox in the lenses were 0.8 γ/g. after 2 hours and 1.2 γ/g. after ½ hour. This is far less than that simultaneously found in erythrocytes, 10 γ/g. and 14 γ/g., respectively.

Discussion

I. General Effects: Nature of Prolonged Acidosis

The experiments reported here present Diamox 2-acetylamino-1,3,4-thiadiazole-5-sulfonamide as a drug with unusual pharmacological properties. In view of the important role of carbonic anhydrase in various regulatory systems in the body, one might expect that inhibition of the enzyme would lead to disaster. Paradoxically, the general toxicity of Diamox proved to be exceedingly low. A further unusual and desirable property is stability in the living organism, as revealed particularly by its nearly quantitative excretion in man. This stability extends to the gastro-intestinal tract, from which Diamox is absorbed unchanged. In common with sulfanilamide and its derivatives, it is partially reabsorbed by the renal tubules; thus the rate of excretion is moderate. Measurable plasma concentrations may be maintained at least 6–12 hours after a single dose.

There appears to be no previous physiologic experience in which systemic acidosis has been maintained and documented for prolonged periods of time. This acidosis, chiefly metabolic but with a respiratory component (Table X, and Ref. 15), has been maintained for 16 months in dogs and 6 months in rats by daily administration of Diamox in high doses. Despite the considerable *chemical* alteration, there was no convincing evidence of toxicity that could

be assigned to the acidosis. There was anorexia and depression in the first month at the highest doses in the dog. These signs, as indicated above, are more likely correlated with potassium depletion, or a combination of acidosis and potassium loss. It is of interest that whatever the cause, the symptoms abated after about a month with no further change evident in body chemistry. It seems that the dog can adapt to his altered internal environment to such degree that his general behavior appears entirely normal. Evidently moderate chemical acidosis with sodium and potassium depletion (see 15 for quantitative data) is itself quite harmless. Whether this is true for man remains to be studied. Symptoms in infantile renal acidosis (45) are anorexia, vomiting, and weight loss, reminiscent of the onset of treatment with high doses of Diamox in the dog. However, as indicated in a recent study of this syndrome, it is not clear that symptoms are attributable to acidosis *per se* (45).

II. RATIO OF ACTIVE TO MAXIMUM TOLERATED DOSE IN DOG

Prior to the discovery of Diamox, it was stated on theoretical grounds that if gastric carbonic anhydrase were inhibited to a measurable degree, the simultaneous effect on CO_2 transport in the blood would be "so seriously affected as to lead to speedy death of the animal" (62). When Janowitz *et al.* (23) did inhibit gastric carbonic anhydrase with Diamox in dogs, this gloomy prediction was not fulfilled. As noted above, Diamox is surprisingly non-toxic, and we have been unable to kill an animal with a single dose up to 2000 mg./kg.

On the basis of the present studies, the margin of safety of Diamox appears to be high. Renal activity can be demonstrated in the dog following 2 mg./kg. and near maximal effects following oral dosage were obtained with 5 mg./kg. (15). Dogs survived 100 mg./kg. day orally with only transient symptoms, as noted; on 33 mg./kg./day they were entirely symptom free.

III. DOSAGE SCHEDULES IN RELATION TO THERAPY

In establishing a rational scheme of treatment for a new drug, it is useful to remember Marshall's (44) warning that a fixed dosage schedule (such as maintenance of more or less constant plasma concentration of drug) is not a satisfactory solution of diversified therapeutic problems. The dosage schedules of Diamox in diseases as diverse as edema in heart failure, epilepsy and glaucoma may be very different. An experimental approach to this problem may be made by studying, in the body, effects of drug on enzyme systems of the organs involved in the various diseases. An attempt in this direction has been made in long term studies of the effect of inhibition of renal carbonic anhydrase on urinary electrolytes. From this experimental work it was possible to suggest a dosage schedule for use in congestive heart failure. This therapeutic plan depends on keeping doses low enough and *spaced far enough apart*, so that there is recovery of acid-base balance between each dose. This

schedule (5 mg./kg./24 hours) is opposite to traditional attempts to maintain a constant plasma concentration of a drug. With the suggested schedule a renal response is obtained following each dose. If, on the other hand, a continuous plasma concentration of Diamox is maintained, no renal response is observed after the first few doses. This illustrates that *opposite* effects may be obtained by variation in dosage schedule (15).

For epilepsy and glaucoma, not enough is known of the mode of action of Diamox, nor have enough schedules been tried clinically to lead to any selection. A reasonable assumption is that in these two disorders, *more or less continuous* maintenance of plasma concentration of Diamox is desired. For epilepsy, this would induce acidosis and some degree of inhibition of enzyme in the brain; however, at the moment one cannot say which or either of these factors is responsible for therapeutic benefit. For glaucoma, maintenance of plasma concentrations of Diamox may be necessary to obtain continuously lowered intraocular pressure.

IV. COMMENTS ON HUMAN TOXICITY

The effects of Diamox in man are not a basic part of this communication. An attempt has been made to show that definitive studies of absorption, distribution and excretion of Diamox in man are still to be done, and that this would be most valuable in connection with electrolyte data.

In closing, however, some attention must be given to reported toxicity of Diamox in man, since human data have imposed limits of dosage which could not be anticipated from the laboratory. The data on animals taken with the usual reservations on species difference would suggest that at very least 33 mg./kg. might be well tolerated in man. From the work documented here, there was little indication what type of toxicity would result from excessive dosage in man. Certain observations in this direction deserve comment.

From the onset of clinical trials, it was evident that man reacted, in at least one aspect, very differently from the animals studied here. At doses above 10–20 mg./kg./day there was usually marked drowsiness, and above 20–30 mg./kg., deep sleep was sometimes encountered (51, 18, 21, 22). All of these signs disappeared on cessation of dosage. It was of interest that sleepiness could not be induced in dogs, rats or monkeys at 25 times the dose that produced it in man. Thus, there is imposed an upper limit on tolerance of Diamox in man, which is far below its lethal range in other species, and probably far below such range in man. The probable therapeutic range in congestive heart disease and epilepsy is 3–10 mg./kg./day (22, 51), and it would appear undesirable to exceed, for the average ambulatory patient, about 15 mg./kg./day.

Two important additional clinical signs which could not, from their nature, be observed in animals also deserve mention. Paresthesias were often noted, particularly tingling and numbing of hands and feet (51, 18, 21, 22). In certain

cirrhotic patients, administration of Diamox was followed by bizarre changes of mood, hallucinations, loss of memory and occasionally psychotic episodes (52). We have not heard of any similar reactions in other types of patients; doubtless this will be subject for future investigation.

Since all of these are central nervous system effects, they are possibly due to reversible inhibition by Diamox of brain carbonic anhydrase. It may also be noted that carbonic anhydrase inhibitors affect transport of potassium in brain slices (53). No explanation for the striking species difference is available, since the enzyme is ubiquitous in its distribution in the brain of different mammals (37). It is of interest that sulfanilamide, the only other carbonic anhydrase inhibitor in clinical use, also produced signs of mental depression in a large percentage of cases (54). It is extremely doubtful if any of these signs are due to potassium loss, since they often occur on the first or second dose, before any considerable loss of potassium could have occurred. Actually, paresthesias and sleepiness are not typical of potassium depletion (56). In some patients, paresthesias occurring in the first 8 hours after dosage declined as plasma concentrations of Diamox could be predicted to be falling off (63). This suggests a direct effect of Diamox on nervous tissue.

Summary and Conclusions

1. A history is given of carbonic anhydrase and its inhibitors, particularly in relation to renal physiology. Studies are listed which led to the development of a powerful and specific inhibitor of carbonic anhydrase, Diamox, 2-acetylamino-1,3,4-thiadiazole-5-sulfonamide.

2. The pharmacology of Diamox was studied extensively in rats and dogs. Limited observations were made in monkeys, guinea pigs, mice and in man.

3. In doses of 20 mg./kg. and less Diamox appears to be rapidly and completely absorbed from the gastrointestinal tract. In dog and in man over 70% of the administered dose is excreted unchanged in the urine by 24 hours. Plasma concentrations of Diamox are proportional to dose, and in the therapeutic range may be detected for 6–12 hours after administration. Renal clearance is approximately two-thirds that of simultaneously administered creatinine in the dog. The drug is distributed in volume corresponding roughly to 40% of body weight in the dog and somewhat less in man. The ratio of the concentrations of Diamox in cerebrospinal fluid or aqueous humor to plasma is approximately 1 to 20.

4. No tissues have special affinity for Diamox. In red cells, however, the drug remains for several days after a single dose. There appears to be a fixed range of concentration which Diamox achieves in the red cell, somewhat independent of dose or length of administration. It is probable that only a small proportion of the carbonic anhydrase in circulating red cells is inhibited by Diamox. No symptoms of retention of CO_2 have been observed.

5. Toxicity of Diamox is very low in the species studied. Using the sodium salt, a single intravenous dose of 2000 mg./kg. failed to kill a dog, whereas the typical renal effect may be elicited with 2 mg./kg. In the mouse, the LD_{50} is between 3000–6000 mg./kg., the same range as sodium lactate or chloride.

6. In long-term studies, dogs tolerated daily oral doses of 100 mg./kg. for 16 months. This is 10–20 times the therapeutic dose. Rats tolerated daily drug-diet doses of 300 mg./kg. for 6 months. At these high doses there was marked metabolic acidosis, and moderate potassium depletion. These were not progressive and did not result in any serious abnormalities during life. There were no definite pathological changes in these animals.

7. In dogs receiving high doses of Diamox for long periods there were no abnormalities in growth, renal clearance, or blood concentrations of glucose, calcium, phosphorus, or sodium. Reproduction was not affected in a variety of species, except for a non-viable litter in a dog at 10–20 times the therapeutic dose.

8. Animal experiments and clinical trials indicate that Diamox is sufficiently non-toxic and that its pharmacological properties are sufficiently desirable to warrant its therapeutic use.

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REFERENCES

1. Meldrum, N. U. and Roughton, F. J. W.: Some properties of carbonic anhydrase, the carbon dioxide enzyme present in blood. J. Physiol., 1932, **75:** 15P.
2. Southworth, H.: Acidosis associated with the administration of para-amino-benzene-sulfonamide (Prontylin). Proc. Soc. Exp. Biol. and Med., 1937, **36:** 58.
2a. Strauss, M. B. and Southworth, H.: Urinary changes due to sulfanilamide administration. Bull. Johns Hopkins Hosp., 1938, **63:** 41.
3. Marshall, E. K., Jr., Cutting, W. C. and Emerson, K., Jr.: The toxicity of sulfanilamide. J. Am. Med. Assn., 1938, **110:** 252.
4. Mann, T. and Keilin, D.: Sulphanilamide as a specific inhibitor of carbonic anhydrase. Nature, 1940, **146:** 164.
5. Höber, R.: Effect of some sulfonamides on renal secretion. Proc. Soc. Exp. Biol. and Med., 1942, **49:** 87.
6. Pitts, R. F. and Alexander, R. S.: The nature of the renal tubular mechanism for acidifying the urine. Am. J. Physiol., 1945, **144:** 239.
6a. Pitts, R. F.: Modern concepts of acid-base regulation. A. M. A. Arch. Int. Med., 1952, **89:** 864.
7. Schwartz, W. B.: The effect of sulfanilamide on salt and water excretion in congestive heart failure. New England J. Med., 1949, **240:** 173.
8. Krebs, H. A.: Inhibition of carbonic anhydrase by sulphonamides. Biochem. J., 1948, **43:** 525.

9. Beckman, W. W., Rossmeisl, E. C., Pettengill, R. B. and Bauer, W.: A study of the effects of sulfanilamide on acid-base metabolism. J. Clin. Inv., 1940, **19:** 635.
10. Davenport, H. W. and Wilhelmi, A. E.: Renal carbonic anhydrase. Proc. Soc. Exp. Biol. and Med., 1941, **48:** 53.
11. Roblin, R. O., Jr. and Clapp, J. W.: The preparation of heterocyclic sulfonamides. J. Am. Chem. Soc., 1950, **72:** 4890.
12. Miller, W. H., Dessert, A. M. and Roblin, R. O., Jr.: Heterocyclic sulfonamides as carbonic anhydrase inhibitors. J. Am. Chem. Soc., 1950, **72:** 4893.
13. Litchfield, J. T., Jr.: Personal Communication.
14. Berliner, R. W., Kennedy, T. J., Jr. and Orloff, J.: Relationship between acidification of the urine and potassium metabolism. Am. J. Med., 1951, **11:** 274.
15. Maren, T. H., Wadsworth, B. C., Yale, E. K. and Alonso, L. G.: Carbonic anhydrase inhibition. III: Effects of Diamox® on electrolyte metabolism. Bull. Johns Hopkins Hosp., 1954, **95:** In press.
16. Davenport, H. W.: Carbonic anhydrase in tissues other than blood. Physiol. Rev., 1946, **26:** 560.
17. Van Goor, H.: Carbonic anhydrase, its properties, distribution and significance for carbon dioxide transport. Enzymologia, 1948–9, **13:** 73.
18. Nadell, J.: The effects of the carbonic anhydrase inhibitor 6063 on electrolytes and acid-base balance in two normal subjects and two patients with respiratory acidosis. J. Clin. Inv., 1952, **32:** 622.
19. Becker, E. L., Hödler, J. E. and Fishman, A. P.: Effect of carbonic anhydrase inhibitor (6063) on arterial-alveolar CO_2 gradient in man. Proc. Soc. Exp. Biol. and Med., 1953, **84:** 193.
20. Tomashefski, J. F., Clark, R. T., Jr. and Chinn, H.: Effect of carbonic anhydrase inhibitor (Diamox) on respiratory gas transport. Fed. Proc., 1953, **12:** 144.
20a. Shepard, R. H., Donoso, H., Killick, E. M., Cherniack, R. M., Johns, C. J. and Riley, R. L.: Interference with release of CO_2 from pulmonary capillary blood after inhibition of carbonic anhydrase. Fed. Proc., 1954, **13:** 135.
21. Friedberg, C. K., Taymor, R., Minor, J. B. and Halpern, M.: The use of Diamox, a carbonic anhydrase inhibitor, as an oral diuretic in patients with congestive heart failure. New England J. Med., 1953, **248:** 883.
22. Belsky, H.: Use of new oral diuretic, Diamox, in congestive heart disease. New England J. Med., 1953, **249:** 140.
23. Janowitz, H. D., Colcher, H. and Hollander, F.: Inhibition of acid secretion in dogs by the carbonic anhydrase inhibitor, 2-acetyl-amino-1,3,4-thiadiazole-5-sulfonamide. Am. J. Physiol., 1952, **171:** 325.
24. Rehm, W. S., Crawford, C. R., Wolf, P. A., Demunbrum, D. O., Hodges, H. and Schlesinger, H. S.: The effect of carbonic anhydrase inhibitor, 6063, on acid secretion and gastric potential. Am. J. Physiol., 1952, **171:** 759.
25. McGowan, J. A., Jr., Stanley, M. M. and Powell, J. J.: The effect of 2-acetylamino-1,3,4-thiadiazole-5-sulfonamide (6063: Diamox) on the secretion of gastric hydrochloric acid in man. (A preliminary report). Bull. New England Med. Center, 1952, **14:** 117.
26. Birnbaum, D. and Hollander, F.: Inhibition of pancreatic secretion by the carbonic anhydrase inhibitor, 2-acetylamino-1,3,4-thiadiazole-5-sulfonamide (Diamox, No. 6063). Am. J. Physiol., 1953, **174:** 191.
27. Becker, B.: Decrease in intraocular pressure in man by a carbonic anhydrase inhibitor, Diamox. Am. J. Ophth., 1954, **37:** 13.

28. Bergstrom, W. K., Garzoli, R. F., Lombroso, C., Davidson, D. T., and Wallace, W. M.: Observations on the metabolic and clinical effects of carbonic-anhydrase inhibitors. Am. J. Dis. Child., 1952, **84:** 771.
29. Westrand, P. J.: Carbonic anhydrase in the anterior uvea of the rabbit. Acta Physiol. Scand., 1951–1952, **24:** 144.
30. Maren, T. H., Ash, V. I. and Bailey, E. M.: A method for determination of carbonic anhydrase inhibitors, particularly of Diamox®. Bull. Johns Hopkins Hosp., 1954, **95:** 244.
31. *War Department Technical Manual* (TM8-227), 1947, p. 196.
32. Ackermann, W. W. and Potter, V. R.: Enzyme inhibition in relation to chemotherapy. Proc. Soc. Exp. Biol. and Med., 1949, **72:** 1.
32a. Chance, B. and Roughton, F. J. W.: Investigations of rates and mechanisms of reactions; *Techniques of Organic Chemistry*, Vol. 8, Interscience Publishers, Inc., New York, 1953, pp. 669–738.
33. *Standard Values in Blood*. Edited by E. C. Albritton, Air Force Technical Report No. 6039, 1951.
34. Mullins, L. J., Fenn, W. O., Noonan, T. R. and Haege, L.: Permeability of erythrocytes to radioactive potassium. Am. J. Physiol., 1941, **135:** 93.
35. Darrow, D. C.: Body fluid physiology: The role of potassium. New England J. Med., 1950, **243:** 978.
36. Streeten, D. M. P. and Williams, E. M. U.: Loss of cellular potassium as a cause of intestinal paralysis in dogs. J. Physiol., 1952, **118:** 149.
37. Ashby, W., Garzoli, R. F. and Schuster, E. M.: Relative distribution patterns of three brain enzymes, carbonic anhydrase, choline esterase and acetyl phosphatase. Am. J. Physiol., 1952, **170:** 116.
38. Vezar, F.: Ability to grow after caloric undernourishment. Helv. Med. Acta, 1940–41, **7:** Suppl. VI; 7.
39. Mackler, B., Lichtenstein, H. and Guest, G. M.: Effect of ammonium chloride acidosis on action of insulin in dogs. Am. J. Physiol., 1951, **166:** 191.
40. Thiersch, J. B.: The failure of Diamox to interfere with reproduction and fetal development in the rat. In Press.
41. Reynolds, S. R. M.: Personal Communication.
42. Albright, F., Burnett, C. H., Parson, W., Reifenstein, E. C. and Roos, A.: Osteomalacia and late rickets. Medicine, 1946, **25:** 399.
43. Henneman, P. and Albright, F.: Personal Communication.
44. Marshall, E. K., Jr.: The dosage schedule of chemotherapeutic agents. Pharm. Rev., 1953, **4:** 85.
45. Lightwood, R., Payne, W. W. and Black, J. A.: Infantile renal acidosis. Pediatrics, 1953, **12:** 628.
46. Heinemann, H. O., and Hödler, J. E.: The effects of 6063 injections on urine and plasma compositions in fishes. The Bull. Mt. Desert Island Biol. Lab., 1953, **4:** 42.
47. Hernandez, T. and Coulson, R. A.: The effect of carbonic anhydrase inhibition on the composition of urine and plasma of the alligator. Science, 1954, **119:** 291.
48. Hendricks, J., Morgan, A. and Freytag, R.: Chronic moderate hypervitaminosis D in young dogs. Am. J. Physiol., 1947, **149:** 319.
49. Mulligan, R. M. and Stricker, E. L.: Metastatic calcification produced in dogs by hypervitaminosis D and haliphagia. Am. J. Path., 1948, **24:** 451.
50. Schmidt, C. L. A. and Greenberg, D. M.: Calcium, magnesium, phosphorus in occurrence, transport and regulation of animal organism. Physiol. Rev., 1935, **15:** 297.

51. DAVIDSON, D.: Personal Communication.
52. EISENMENGER, W.: Personal Communication.
53. DAVIES, R. E. AND KREBS, H. A.: Biochemical aspects of the transport of ions by nervous tissue. Biochem. Soc. Symposia, 1952, **8:** 77.
54. NORTHEY, E. H.: *The Sulfonamides and Allied Compounds*, Reinhold Publishing Corp., New York 1948, p. 565.
55. FISHER, S. H., TROAST, L., WATERHOUSE, A. AND SHANNON, J. A.: The relation between chemical structure and pharmacological disposition of a series of substances allied to sulfanilamide. J. Pharmacol. and Exp. Therap., 1943, **79:** 373.
56. FOURMAN, P.: Depletion of potassium induced in man with an exchange resin. Clin. Science, 1954, **13:** 93.
57. JAFFE, H. L., BODANSKY, A. AND CHANDLER, J. P.: Ammonium chloride decalcification as modified by calcium intake. J. Exp. Med., 1932, **56:** 823.
58. WALTON, R. L. AND BERGSTROM, W. K.: Effects of Diamox on electrolyte excretion and body composition of the rat. Presented at the Meeting of the Society for Pediatric Research, May 1954.
59. BALLINTINE, E. J. AND MAREN, T. H.: Unpublished Observations.
60. RELMAN, A. S., LEAF, A. AND SCHWARTZ, W. B.: Oral administration of a potent carbonic anhydrase inhibitor (Diamox). II. Its use as a diuretic in severe congestive heart failure. New England J. Med., 1954, **250:** 800.
61. MARTIN, G. J., BALANT, C. P., AVAKIAN, S. AND BEILER, J. M.: Inhibition of carbonic anhydrase. Arch. Internat. Pharmacod., 1954, **98:** 284.
62. DAVIES, R. E.: Hydrochloric acid production by isolated gastric mucosa. Biochem. J. 1948, **42:** 609.
63. LANGFORD, H. G.: Personal Communication.

CARBONIC ANHYDRASE INHIBITION

II. A Method for Determination of Carbonic Anhydrase Inhibitors, Particularly of Diamox®

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The background for study of carbonic anhydrase and its inhibitors in relation to electrolyte physiology has been described (1). With the development of powerful inhibitors of this enzyme (2, 3) it was essential to carry out pharmacological investigations prior to and coincident with metabolic and therapeutic studies. Determinations of Diamox 2-acetylamino-1,3,4-thiadiazole in the preceding pharmacological study (1); were done by the biological method reported below.

TRIALS OF CHEMICAL METHODS

Soon after it appeared that Diamox had interesting physiological properties, we sought chemical methods for its determination in biological fluids. These were all unsuccessful, although they throw some light on the chemical reactivity of Diamox. Brief note will be made of these experiments with views both toward preventing duplication elsewhere and stimulating efforts which may be more fruitful.

1. Diazotization and Coupling. 2-Amino-1,3,4-thiadiazole-5-sulfonamide was used throughout. The conventional Bratton and Marshall procedure (4) gave no color with 100γ. Variations in the method, specifically with regard to acid strength and temperature, also failed. Coupling in alkaline solution with hexylresorcinol (5) was equally fruitless. The compound did not give a color with diazotized sulfanilic acid (6). Finally it failed to give a color with Ehrlich's reagent, *p*-dimethylaminobenzaldehyde (7).

2. Reaction with Folin's Reagent, Sodium β-naphthoquinone-4-sulfonate. 2-Amino-1,3,4-thiadiazole-5-sulfonamide was used, in aqueous stock solution containing 20 γ/ml. Optimal conditions for color formation follow: Two ml. of stock solution were added to 6 ml. buffer at pH 12.4 (1 ml. of 6 M NaOH plus 5 ml. of 0.6 M KH_2PO_4) followed by 1 ml. of Folin's (8, 8a) reagent (1 mg./ml. in water). A salmon pink color developed in about 15 minutes. Alternatively, the compound could be extracted from acidified stock solution into butanol, returned to the alkaline buffer, and Folin's reagent added. Both procedures were limited by the interference of biological materials and the instability of the final color.

3. Ultraviolet Absorption. The UV absorption peak of Diamox in water is at 267 mμ; that of 2-amino-1,3,4-thiadiazole-5-sulfonamide is at 277 mμ. The molar extinction coefficients

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were, respectively, 10,690 and 7860. (UV data from R. C. Hirt). In a limited series of experiments with the latter compound, it did not appear that its extraction characteristics into common organic solvents were such that it could be readily separated from biological material and measured in the UV with the desired sensitivity.

4. Degradation to Hydrazine. It was found, following a suggestion of Dr. R. O. Roblin, Jr., that under rather extreme conditions of temperature and acidity, the thiadiazole ring in 2-acetylamino-1,3,4-thiadiazole-5-sulfonamide could be degraded to yield hydrazine. In 27 N H_2SO_4 at 200°C. this reaction was quantitative in 1 hour. The reaction mixture was partially neutralized and a solution of *p*-dimethylaminobenzaldehyde in ethanol and HCl was added. An intense orange-yellow color formed, due to an azine that rearranges in acid solution to a quinoid structure (9). It was possible to detect 1γ of hydrazine or the equivalent of 7γ of Diamox. Unfortunately, this technique could not be applied to biological material; it appeared as if hydrazine, after being split from Diamox, reacted irreversibly with tissue constituents of the digest and was not free to react to give the azine. Attempts to extract Diamox from water or tissue into organic solvents and run the thiadiazole degradation on tissue-free extracts were generally unsuccessful.

METHOD UTILIZING CARBONIC ANHYDRASE

General

The procedure is essentially the changing pH method of Philpot and Philpot (10), modified by Garzoli (11) to give greater accuracy by the introduction of photoelectric determination of end-point and a reaction vessel to provide automatic introduction of CO_2 and evacuation of fluid. The principle follows: CO_2 is bubbled at constant rate through water containing indicator dye which changes color at pH *circa* 7. A standard amount of buffer (pH 9) is then added, causing the indicator to change color. The hydration of CO_2 to form H_2CO_3 neutralizes the added buffer base and the indicator turns back to the original (acid) color. In the system to be described below, the time (measured from

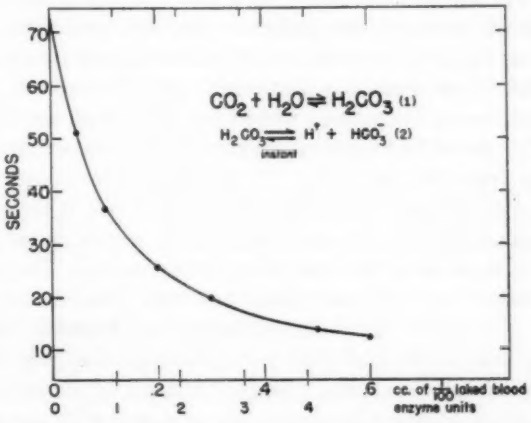

FIG. 1. The effect of carbonic anhydrase on the rate of hydration of carbon dioxide.

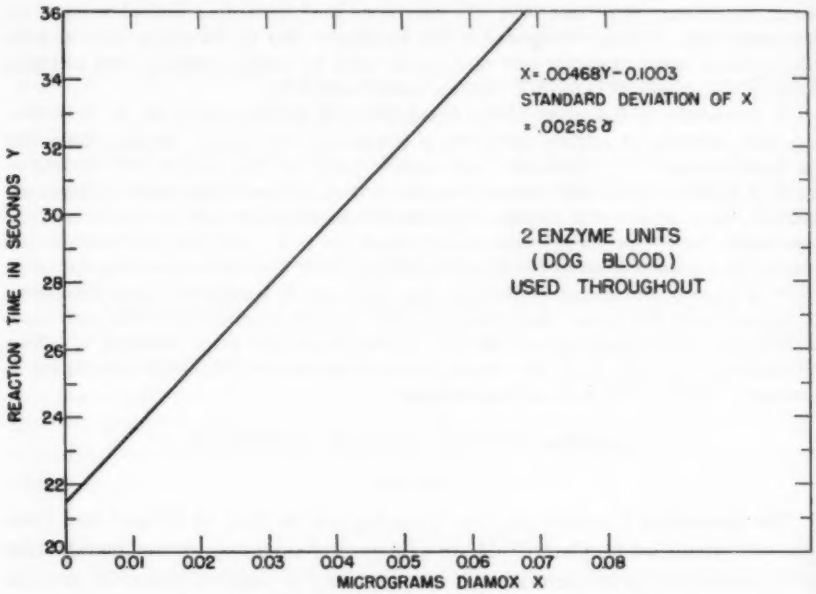

FIG. 2. Standardization graph for Diamox analysis.

the addition of buffer) of hydration is 62–72 seconds in the absence of carbonic anhydrase. This range of uncatalyzed reaction time may be achieved by adjustment of flow of CO_2. When the enzyme is added, the reaction rate increases and there is a reproducible relationship between time and enzyme concentration (Fig. 1). One enzyme unit is defined as the quantity which will double the rate of the uncatalyzed reaction under the specified conditions.

To determine inhibitor, enzyme concentration is kept constant. Two units of carbonic anhydrase from dog blood were used throughout. As shown in Figure 2 (see below for discussion), within a certain range the time of reaction is then directly related to concentration of inhibitor present. As indicated in a previous study from this laboratory (3), this general technique is suitable for a wide variety of carbonic anhydrase inhibitors. Since it measures only inhibition of enzyme activity, the question must at once be raised as to whether this is the true measure of the compound being studied. The identity of inhibitory "activity" with Diamox itself has been proved independently in pharmacological studies; after administration of Diamox, the "activity" of dog urine in this enzyme inhibition test agrees quantitatively with recovered and recrystallized drug (1). Thus, it seemed suitable to use the inhibition test as the means of determining Diamox in the dog, and with some reliability in other species (1).

Details of the Test

a. Apparatus. The basic design of our apparatus was copied from Garzoli's titrimeter (11), which was kindly loaned to us. Our equipment incorporates improvements, such as a higher voltage lamp and adjustable transformer to give a steadier light, a more stable electronic circuit, a more sensitive system for study of turbid samples, changes for greater electrical safety, and differences of construction. However, the two instruments are sufficiently similar to produce analyses which are in good agreement.

The entire apparatus consists of a glass reaction vessel of the dimensions of a standard test tube, with a side arm for introduction of CO_2 and a capillary outlet tube on the bottom for evacuation; a lamp which shines a focussed beam through the reaction vessel and through two color filters to a phototube; a refrigerated constant-temperature bath (American Instrument Co.) containing ethylene glycol and water at 0.4°C.; an electronic amplifier; a relay operated by the amplified output of the phototube; and a timer operated by the relay. The brightness of the lamp is manually adjusted by a Variac to give an appropriate initial reading on an ammeter which is located in the output circuit of the amplifier. Figure 3 shows the electrical circuit, which derives originally from that due to Shepard (12). Figure 4 shows the phototube, lamp, and reaction vessel assembly, which is immersed in the constant-temperature bath.

The apparatus operates as follows: Initially, CO_2 bubbling through the phenol red solution in the reaction vessel lowers the pH and the indicator turns yellow. When alkaline buffer is added, the indicator turns red and the reaction starts. The red solution, in conjunction with the filters, reduces the light reaching the phototube. This lowers the current in the relay in the output circuit of the phototube amplifier and *starts* the timer. As the reaction proceeds the pH drops, changing the indicator from red to yellow. This increases the light reaching the phototube and reactivates the relay, *stopping* the timer. The elapsed time is that of the hydration of CO_2; as noted elsewhere in the text, this time may be related to concentrations of added carbonic anhydrase (Fig. 1) or inhibitor (Fig. 2) under appropriate conditions.

Figure 4. The Reaction Assembly. Since the reaction vessel is immersed in the bath, the lamp and phototube are also immersed to avoid the effect of surface ripples on the light beam. Glass jars protect the lamp and phototube from the water. All optical parts must be rigidly mounted with respect to one another because of the sensitivity of the amplifier. Ventilation was originally provided for the lamp, but water condensed inside the cool jar. The vents were closed to stop the intake of air, adequate cooling is provided by the bath. Only light passing through the sample reaches the phototube, which is protected from extraneous light, and shielded electrically. The phototube is mounted close to the reaction cell to intercept light scattered by turbid samples. A bag of calcium chloride is kept in the jar to reduce moisture and to maintain the high impedance of the phototube. Two glass filters are mounted directly in front of the phototubes. These combine to make a green filter, which will not pass magenta

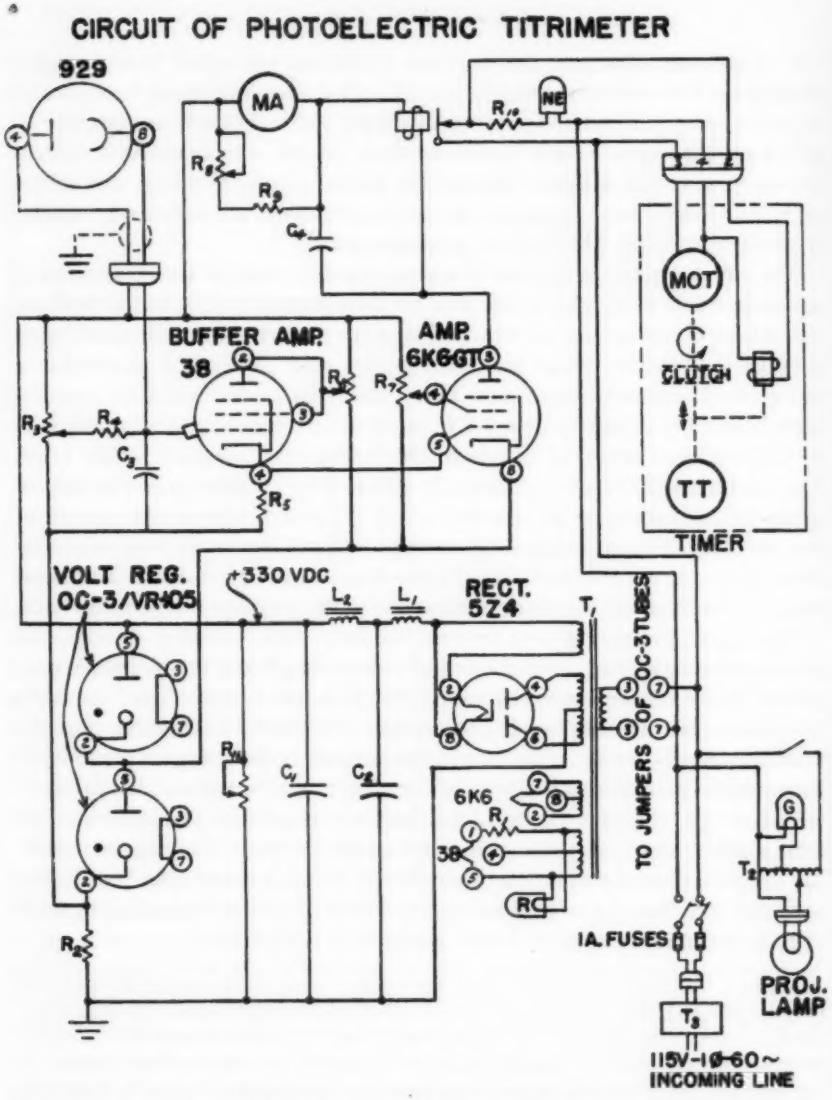

FIG. 3. R_1—Resistor, 10 ohms, 5 watts. R_2—Resistor, 10,000 ohms, 25 watts, adjustable. Left set at 3,800 ohms to give good voltage regulation and to keep OC-3's glowing. R_3—Potentiometer, 1 megohm, 2 watts. R_4—Resistor, 18 megohms, ½ watt. R_5—Resistor, 470,000 ohms, 2 watts. R_6, R_7—Potentiometers, 100,000 ohms, 2 watts. R_8—Potentiometer, 25 ohms, 2 watts. R_9—Resistor, 10 ohms, ½ watt. R_{10}—Resistor, 1 megohm, ½ watt. R_{11}—Resistor, 18,000 ohms, 25 watts. C_1, C_2—Condenser, electrolytic, 8μf., 600 d.c. working volts. (Aerovox Type GL). C_3—Condenser, 10,000 μμf. C_4—Condenser, 2 μf. L_1—Choke, 10 H.,

light from the alkaline sample, but which passes yellow light when the reaction turns acid. The combined curve of spectral emission of the lamp and of spectral sensitivity of the phototube has a maximum near the operating point of the color filters (545 milli-microns). Although changing the brightness of the lamp by means of the Variac to compensate for different sample opacities must change the color of the emitted light, no effect has been observed on the measurements, probably because the lamp-phototube curve is rather broad, the color change of the lamp is slight and the color change of the indicator at the end point is rapid.

Figure 3. The Electrical Circuit. This includes a power supply, phototube, two-stage amplifier, milliammeter, relay, timer, projection lamp, and Variac.

The power supply consists of power transformer T_1, a 5Z4 full wave rectifier, a choke-input smoothing filter, and two OC-3 voltage regulating gas tubes. The design of the power supply is conventional and conservative for safety and good smoothing. The choke-input type of filter, bleeder resistor R_{11} and 600 volt condenser ratings protect against excessive peak voltages which might occur under certain circumstances. The bleeder also discharges the condensers in a few seconds when the power is switched off, a safety feature.

The division of voltage between the phototube and its load resistor, R_4, depends on illumination. The resulting signal is applied to the grid of the RCA 38 pentode which operates in this circuit as a buffer amplifier with a high grid impedance to match the impedance of the phototube circuit. The phototube, its lead-wires, and the amplifier are protected from electrical interference by grounded shielding. The buffer amplifier is cathode-coupled to the 6K6-GT power pentode (the second amplifier stage), in the plate circuit of which are the coil of the relay and the milliammeter. The meter has a variable shunt for adjusting its sensitivity with respect to that of the relay. The relay controls the timer and neon pilot lamp, which derive their power from the line. The timer contains a synchronous motor which runs continuously to avoid starting inertia and which is connected to clock dial TT by means of a built-in solenoid-operated clutch.

The projection lamp is a 120 volt type rather than the more usual 6 volt lamp which is generally thought to have more stable intensity (perhaps because it is often used with storage batteries). We find instead that the 120 volt lamp is more stable, presumably because lower current at the higher voltage causes less heating and corrosion of connections, higher lamp resistance makes changes in contact resistances less noticeable, and the sliding contact of the

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200 ma. L_2—Choke, 8 H., 120 ma. NE—Neon pilot light (Westinghouse NE-51 bulb). G—Pilot light, green lens, incandescent bulb 28V (GE Mazda 313). R—Pilot light, red lens, incandescent bulb 6 to 8V. MA—Milliammeter, 0–1 ma. d.c. (Weston Model 301). Proj. Lamp—75 W, 120V, S.C. base (GE Code—75T8/72 projection bulb). Timer—The Standard Electric Time Co., Springfield, Mass. Type S-10, Movement TI, 6 r.p.m., 115V, 60 c.p.s., 0 to 1000 sec., one small division equals 0.1 sec. All parts inside dashed line are included in timer as purchased. TT—Clock dial indicating total elapsed time. T_1—Power transformer, 375 volts r.m.s. each side of center, 100 ma.; 5V-3A; 6.3 VCT-4A; 6.3 VCT-2A. (U.T.C. R-12). T_2—Autotransformer, adjustable output 0-135V; input 115V, 1A., 60 c.p.s. ("Variac 200B", Gen. Radio Co.). T_3—Transformer, voltage regulating; primary—95 to 125V., 120 V.A., 60 c.p.s.; secondary—115V., 1.04A. (Sola Electric Co., Cat. 30806). 929—Vacuum phototube (R.C.A.). 38—Pentode vacuum tube (R.C.A.). 6K6-GT—Pentode vacuum tube (R.C.A.). OC-3/VR-105—Gaseous voltage regulator tube. (R.C.A.). 5Z4—Rectifier tube (R.C.A.). Relay—Coil 1 ma., d.c.; contacts 115V. d.c. 0.25A. (Struthers Dunn, Inc., Phila., 112 XAX).

Numbers on tubes in diagram refer to manufacturers type.

IRE standard graphical symbols (13) were used on this diagram.

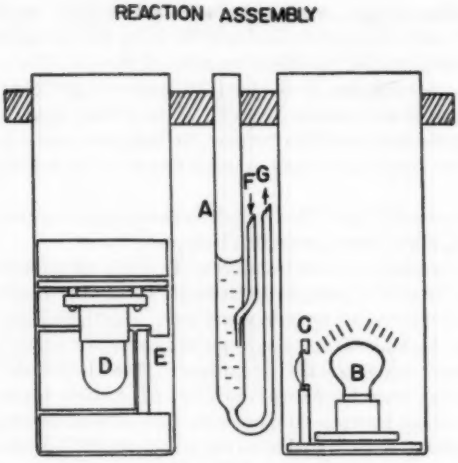

FIG. 4. A. Reaction vessel, 20 × 150 mm. F. Inlet for CO_2. The gas is delivered at about 600 ml./min from a tank to a sintered glass bubbler in a wash bottle partially filled with water and immersed in the ethylene glycol bath at 0.4° C. It is then delivered to F, a capillary tube 2 mm. inside diameter which ends in a tip about 1 mm. inside diameter within A, and extending ½–⅔ of the distance between the surface of the liquid and the bottom of the tube. G. Capillary (2 mm. inside diameter) outlet to aspirator. A stopcock between the capillary and aspirator is closed during the reaction and opened for evacuation of the contents of the tube. B. Projection lamp, see legend of Figure 3. C. Condensing lens, Edmund Scientific Corp. No. 1059, diameter 1 3/16 in., focal length 1¼. D. Phototube, see legend of Figure 3. E. Corning color filters, No. 3486 "H. R. Yellow Shade Yellow" and No. 9782 "Blue-Green".

The housings for the lamp, lens, photocell and filter are painted glass jars, with watertight fittings at the top.

This is not a scale diagram.

Variac used with the 120 volt lamp may not get as hot as that of the rheostat which adjusts the 6 volt lamp customarily used.

The main purpose of the voltage regulating transformer, T_3, is to supply constant voltage to the lamp, but it also gives further voltage regulation to the rest of the circuit. Transformer T_3 should be disconnected at its input when not in use as it draws nearly full current even with the secondary switch open.

The electronic circuit and its cabinet, the case of transformer T_3, the water bath, stirring motors, etc. should all be connected to a common ground (water pipe) for safety and polarized plugs should be used to prevent crossed circuits.

It was found that the bubbling CO_2 gives adequate mixing for the reaction without serious interference with the light beam. Relatively turbid samples can be measured because of the powerful 75 watt lamp and the condensing lens. Condensers C_3 and C_4 further reduce meter fluctuations and eliminate relay chatter, which caused arcing and sticking of the relay contact. For the same reason, ripple was reduced by the better filter shown in Figure 3.

Condenser C_3 cannot be extremely large without slowing the speed of response to the signal. When C_3 is 10,000 $\mu\mu$f., response is rapid and the remaining meter fluctuations have negligible effect on the measurements.

b. Operation of the Titrimeter

To turn on the instrument, turn on the power switch 2 minutes before turning on the lamp. Steps 1–7 are permanent settings which are checked about once a year.

1. Add 5.0 milliliters indicator solution and 2.0 milliliters water to reaction vessel.
2. Set potentiometers R_3 (control grid voltage of the first amplifier stage), R_6 (plate and screen voltage of the first stage), and R_7 (screen voltage of the second stage) to the high voltage end of their ranges and R_8 to the low resistance (low meter sensitivity) end of its range.
3. Turn R_7 until a reading of 0.7 ma. on the meter is reached. Steps 2 and 3 serve to fix the maximum relay current possible (high phototube illumination). This current should exceed that needed to pull in the relay. When meter sensitivity is restored by Step 7 below, this maximum current will be somewhat above the range of the meter.
4. Turn R_6 until meter reads 0.68 ma. This slight movement of the meter occurs when the contact of R_6 has been moved just far enough negative to bias the grid of the 6K6 slightly negative with respect to its cathode.
5. Turn off lamp.
6. Turn R_3 until the meter reaches 0.02 ma., one small scale division above zero. This part of the scale now represents zero phototube illumination.
7. Turn R_8 back to full meter sensitivity. The instrument is now ready and sensitive to light changes, provided the light has the proper intensity, neither too high nor too low.
8. Operation of the titrimeter in each reaction is described below (under e).

c. Care of the Reaction Vessel

The reaction vessel is cleaned with detergent (Vel) and hot water twice daily, using a test-tube brush. Between reactions the vessel is rinsed three times with distilled water. To prevent foaming, a small drop of DC Antifoam A (Dow Corning) may be added to the vessel. The effect of this emulsion lasts through 20–30 determinations.

d. Solutions. All solutions are kept in the constant-temperature bath before use.

Indicator. 12.5 milligrams of phenol red are dissolved in 1 liter of .0026 M $NaHCO_3$. Five milliliters are used in each determination.

Enzyme. Heparinized dog blood is added to water, 1:100. In our hands, 0.24 milliliter of this suspension is equivalent to 2 enzyme units. Such a suspension appears to be stable at 4°C. for approximately 4 months. More dilute suspensions are definitely less stable.

Buffer. 300 milliliters of 1 M Na_2CO_3 are added to 206 milliliters of 1 M $NaHCO_3$ and made up to 1 liter. One milliliter is used.

Sample. Biological material is diluted or ground with water, usually to give a final concentration of 1:50. The suspension is placed in a water bath at 100°C. for 5 minutes. This destroys any carbonic anhydrase that may be present, but does not degrade Diamox. No more than 0.75 milliliter of such a suspension usually is used in one determination.

e. Procedure for Determining Inhibitor. Carbon dioxide is bubbled through water in a bottle immersed in the constant temperature bath and led to the

reaction vessel at a constant rate. (A flowmeter designed by Brooks Rotameter Company, Lansdale, Penna., No. 2–15–2, was used to monitor the flow.) Five milliliters of the phenol red solution is added and then 2 units of carbonic anhydrase. The standard or the unknown (after dilution and boiling) is added and then water to make 6 milliliters in the reaction vessel. For the last two additions pipettes calibrated to .01 milliliter are used. At this stage the milliammeter is set to a standard predetermined value (we use 0.9 ma.) and the timer is read or set to zero. One milliliter of buffer is added. Instantly the solution turns red and the milliammeter reading falls, starting the timer. The timer stops when the solution turns yellow, giving the time of hydration of CO_2 over the pH range of phenol red. From the calibration curve (Fig. 2) and the dilution factor, the concentration of Diamox can be simply calculated. After a determination, the reaction vessel is evacuated and rinsed.

Each determination is run rapidly, with no wait between any of the steps. It is desirable to reproduce each maneuver as closely as possible on each run. Under these conditions equilibrium between Diamox and carbonic anhydrase is not reached; if this is desired, inhibitor and enzyme should stand for 3 minutes at room temperature or approximately 1 hour at 0°C.

f. Procedure for Determining Enzyme. The technique is similar to that just described, except that the unknown is the unboiled enzyme-containing material. It is added to the phenol red, then the volume is made up to 6 milliliters and buffer added. The standard curve of Figure 1 is used to relate time to enzyme concentration. ***Determination of enzyme concentration cannot be made directly in the presence of inhibitor.*** By indirect methods (1), we have shown the approximate degree of enzyme activity in the blood of dogs receiving Diamox.

EVALUATION OF THE METHOD FOR DETERMINATION OF DIAMOX

No interference with the method was encountered. Normal biological tissue from rat and dog does not appear to contain any inhibitor detectable by this technique. Thus no false positives were found; tissues from untreated controls invariably gave a reaction time under 23 seconds, the same as "blank" without tissue (Table IA, IC). The other potential source of interference, tissue or blood enzyme, is entirely destroyed by the heating procedure.

Diamox, as has been noted, is unusually stable (1). It withstands brief heating at 100°C. and appears stable at 4°C. whether in aqueous solution or in tissues. Table I shows repeat assays over the course of several weeks, to illustrate the range of the method, both for the standardization curve (Table IA and Fig. 2) and for the extent of reproducibility of assays on tissue samples (Table IB).

It will be observed that the method is usable only in the range of .01–.06 microgram in the sample. This is not a serious limitation, since the size of the sample may be varied between .05–.75 milliliter to get the working range.

TABLE I

(A) Data for Standardization for the Determination of Diamox

Diamox γ Added	Reaction Time in Seconds						Average ± S.D.
	Day 1	Day 1	Day 1	Day 2	Day 3	Day 4	
0.0	21.0	21.8	20.8	22.5	22.3	21.2	21.60 ± 0.71
0.01	23.6	22.8	23.0	24.2	23.6	23.5	23.45 ± 0.50
0.02	24.7	25.5	25.8	25.8	25.3	25.6	25.45 ± 0.41
0.04	29.1	30.5	30.0	29.9	30.8	30.4	30.12 ± 0.60
0.06	34.9	34.0	33.6	34.5	34.4	33.9	34.22 ± 0.47
0.08	35.6	34.7	34.8	—	—	—	—

(B) Illustrating Reaction Times of Repeat Analyses on Organs of Treated Rats*

Rat No.	Organ	Volume	Seconds				
			11/5/52		11/26/52		
		ml.					
28	Liver	0.5	27.2	26.7	26.1		
62		0.5	30.0		30.8	30.5	31.0
28	Kidney	0.1	26.3	26.9	26.7		
19		0.1	34.4	33.9	35.6	34.9	
62		0.1	26.1	26.1	26.3		
28	Pancreas	0.1	33.0	32.6	32.6	32.9	
19		0.2	33.0	32.4	33.6	34.1	
9		0.5	25.3	26.0	26.0		

(C) Illustrating Reaction Times of Organ Suspensions of Untreated Rat†

Organ	Time	Organ	Time
	sec.		sec.
Liver	21.6	Lung	22.1
Kidney	22.4	Spleen	20.1
Muscle	20.8	Brain	20.3
Plasma	21.1	Adrenal	22.8

* Data from experiment of Table IX in (1). Sacrificed 10/22/52. All samples 1:50 dilution.
† 0.2 milliliter of 1:100 suspension used.
Tissue in (B) and (C) boiled to destroy enzyme, as described in text.

Also, the dilution may be altered, the only limit being the opacity of the final solution. In our hands this begins to interfere with light transmission when the dilution of tissue sample is of the order of 1:25. This sets the lower limit of the method for tissue at approximately 0.5–1.0 microgram of Diamox per gram of tissue, the situation where dilutions of 1:50 are used. For plasma and urine dilutions of 1:20 are possible, and the lower limit is about 0.2 microgram per ml. of sample. As each determination takes but several minutes, adjustment of sample size or dilution does not seriously hinder the procedure.

Table II is representative of recoveries obtained when known quantities

TABLE II

"Recovery" of Diamox Added to Rat Tissues

Tissue	Amount of Whole Tissue	Conc. of Added Diamox Calc. for Whole Tissue	"Recovery"
Plasma	0.5 ml.	10 γ/ml.	10.0 γ/ml.
Erythrocytes	0.7 ml.	10 γ/ml.	10.5 γ/ml.
Kidney	1.21 g.	10 γ/g.	10.5 γ/g.
Liver	1.87 g.	10 γ/g.	8.8 γ/g.
Lung	0.46 g.	10 γ/g.	9.0 γ/g.
Muscle	0.65 g.	10 γ/g.	10.0 γ/g.

Tissues from an untreated rat were ground with water and diluted to give a final suspension of 1:50, each ml. of which contained 0.2 γ added Diamox. From 0.1–0.2 ml. of this suspension was used in the determination, carried out as described in the text. The amount of Diamox present was read off the curve (Fig. 2) in the usual manner, and multiplied by the appropriate dilution factors to give the figure in the last column. Readings were in the range 27–37 seconds.

of Diamox are added to tissue homogenates and carried through the procedure outlined above. As noted above, normal tissues invariably give a 0 reading for drug. Since there is no detectable inhibitor in control tissue and since enzyme is destroyed in the procedure, values obtained in tissue homogenates are in no way different from those found in aqueous solution. Figure 2 gives the calibration curve fitted by the method of least squares applied to the data of Table IA, excluding 0.08 microgram, which is beyond linear range. Analysis of variance indicated no significant difference between runs of the several days. The standard deviation of a single determination is on the average $\pm 10\%$ of the concentration of Diamox (Fig. 2).

SUMMARY

1. The changing pH method for determination of carbonic anhydrase activity has been adapted for study of inhibitors of this enzyme. Since there is no inhibitor normally present in tissue and since after *in vivo* administration recovery of Diamox by gravimetric and enzymatic methods agree, the latter measurement appears equivalent to determination of the compound itself.

2. The method is sufficiently sensitive (0.5 microgram per gram tissue) and accurate ($\pm 10\%$) for determination of Diamox in biological samples.

3. Unsuccessful attempts to develop a chemical method are briefly recounted, since they throw some light on the reactivity of 2-amino-1,3,4-thiadiazole-5-sulfonamide in comparison to that of certain arylamines.

ACKNOWLEDGMENT

We wish to thank Mr. Ray F. Garzoli and Dr. Eliot Wannamaker of the Wannamaker Chemical Company for the use of their photoelectric titrimeter during the early stage of this work, and for Mr. Garzoli's consultation.

REFERENCES

1. MAREN, T. H., MAYER, E., AND WADSWORTH, B. C.: The pharmacology of Diamox® 2-acetylamino-1,3,4-thiadiazole-5-sulfonamide. Bull. Johns Hopkins Hosp., 1954, **95:** 199.
2. ROBLIN, R. O., JR. AND CLAPP, J. W.: The preparation of heterocyclic sulfonamides. J. Am. Chem. Soc., 1950, **72:** 4890.
3. MILLER, W. H., DESSERT, A. M. AND ROBLIN, R. O., JR.: Heterocyclic sulfonamides as carbonic anhydrase inhibitors. *ibid.*, 1950, **72:** 4893.
4. BRATTON, A. C. AND MARSHALL, E. K., JR.: A new coupling component for sulfanilamide determination. J. Biol. Chem., 1939, **128:** 537.
5. MAREN, T. H.: The fate of two arsenoxides in the dog. J. Pharmacol. and Exp. Therap., 1949, **96:** 250.
6. BRODIE, B. B., UDENFRIEND, S. AND TAGGART, J. V.: The estimation of basic organic compounds in biological material. IV. Estimation by coupling with diazonium salts. J. Biol. Chem., 1947, **168:** 327.
7. WERNER, A. E. A.: Estimation of sulfanilamide in biological fluids. Lancet, 1939, **1:** 18.
8. FOLIN, O.: A colorimetric determination of the amino acid nitrogen in normal urine. J. Biol. Chem., 1922, **51:** 393.

8a. SCHMIDT, E. G.: The determination of sulfanilamide in tungstic acid blood filtrates by means of sodium β-naphthoquinone-4-sulfonate. J. Biol. Chem., 1938, **122:** 757.

9. AUDRIETH, L. F. AND OGG, B. A.: *The Chemistry of Hydrazine.* John Wiley & Sons, New York, N. Y., 1951, p. 164.
10. PHILPOT, F. J. AND PHILPOT, J. ST. L.: A modified colorimetric estimation of carbonic anhydrase. Biochem. J., 1936, **30:** 2191.
11. GARZOLI, R. F.: Personal Communication.
12. SHEPARD, F. H., JR.: In RCA Phototubes, RCA Mfg. Co., 1940, p. 10.
13. *IRE Standard on Graphical Symbols for Electrical Diagrams*, Proc. I.R.E., 1954, **42:** 965–1020.

PHYSIOLOGICAL STUDY OF THE MONKEY FETUS IN UTERO: A PROCEDURE FOR BLOOD PRESSURE RECORDING, BLOOD SAMPLING, AND INJECTION OF THE FETUS UNDER NORMAL CONDITIONS

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An ideal study of fetal and placental physiology ought to be made with the fetus *in utero* and the membranes intact. Only in this way may it be said that pressure and vascular relationships within and about the fetal vascular system are normal as the uterus contracts and relaxes normally. Investigation of the fetal circulation usually involves delivery of the fetus by Caesarean section with the placental circulation maintained, and breathing prevented by pulling a rubber sheath over the head at the moment of delivery (Barcroft, 1947; Ardran, Dawes, *et al.*, 1952; Huggett, 1927; Reynolds, 1953). While this maneuver can be carried out well in some animals such as the ewe and the goat, it cannot be done readily in monkeys since the uterus contracts following reduction of volume as the fetus is delivered and placental separation results.

Studies of placental transfer of substances in monkeys required that a procedure be developed whereby the foregoing consequences might be circumvented. It was necessary that the volume of the uterus be retained, and if possible, that there be no rupture of the amniotic sac, that the fetus remain *in utero*, and that there be minimal operative trauma inflicted upon the uterus. Such a technic and preliminary results with its use are reported in this paper.

MORPHOLOGICAL BASIS OF TECHNIC

The basis of the procedure described is the fact that the monkey has two placentas, one a large, primary structure which receives the umbilical cord, the other a secondary one which receives its blood supply from two to four vascular extensions of the umbilical vessels running along the chorio-allantoic surface of the primary placenta. These relations are shown in Figure 1.

These interplacental vessels pass between the two enveloping membranes of the conceptus, the amnion and the chorion which, filled with the fetus and fluid of the conceptus, distend the uterus. In this extra-amniotic position, a short segment of the vessel may be localized, delivered, cannulated and cathe-

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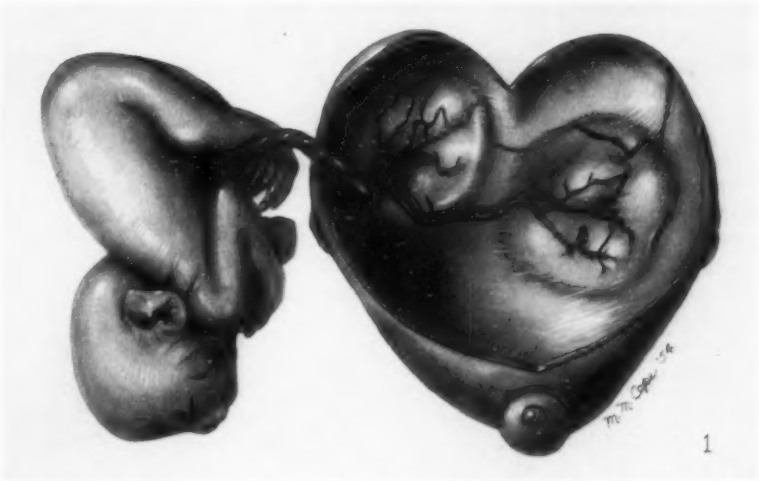


FIG. 1. Morphological relations in the monkey of the primary placenta (receiving the umbilical cord), the secondary placenta and the interplacental vessels connecting them. The latter lie outside the amnion, and beneath the chorion. In this position they can be localized, a segment delivered, and catheterized.

terized. Such a procedure permits recording of fetal blood pressure and fetal heart rate, taking samples of blood, and injection of materials directly into the fetal circulation, all while the fetus is still *in utero* and contained within the amniotic sac.

In about one monkey out of five, the primary and secondary placentas are contiguous, either as a large plaque-like structure, or with the secondary placenta lying as a band of tissue stretching around the uterus and touching the primary placenta at two points. In each instance the interplacental vessels are short and lie wholly upon the chorio-allantoic surfaces of the two placentas rather than between the membranes adjacent to the uterine wall. There the vessels are inaccessible to operative interference. The problem is to recognize the situation early in the experiment so as to change the experimental design into a useful alternative procedure or to close the monkey for later use.

With slight modification, we know the procedures to be described below to be applicable to the cotyledonary type of placenta of the sheep and goat, for we have applied them successfully in two pregnant ewes.

METHODS

Animals. Eleven monkeys have been used. In one monkey, ninety-two days pregnant, an interplacental vessel was cannulated with the smallest sized catheter available, but blood samples could not be readily obtained through it. No attempt was made to record blood pressure. In two monkeys, the primary and secondary placentas touched in the manner

noted above. In these, therefore, the fetal head was delivered through a small uterine incision, and excessive loss of fluid was prevented by a purse-string suture which approximated the margin of the cut uterus to the neck of the fetus. Blood samples were then taken by puncture from the sagittal sinus. The remaining animals, eight in number, were subjected to cannulation of the interplacental arteries. The duration of pregnancy in these ranged from about 146–156 days. Gestation in rhesus monkeys lasts approximately 168 days.

Anesthesia. After the first monkey, in which Nembutal was given (0.2 ml. per kilogram) by vein, Pentothal anesthesia was administered by way of the saphenous vein. This vessel was catheterized, and Pentothal (12.5 mg./ml.) was administered usually in 0.5–1.0 ml. amounts as needed throughout the experiment. The total quantity of anesthetic has amounted to 14 ml. given over a period of more than three hours.

Materials and instruments. The only special items required for exposure and cannulation of the interplacental vessels are the following: small, fine-pointed mosquito clamps, polyethylene tubing (1.1 mm. O. D.; 0.8 mm. I. D.), two pocket flashlights, fine scissors, a very small bull-dog clamp; a number 18 needle, the point of which is ground off and fitted into the tubing; a three-way adapter stopcock, heparin solution in a syringe. For recording blood pressure, a Statham pressure transducer (2.5 p.s.i.) has been used, attached to the three-way stopcock, and so to a suitable ink-writing recorder (Brown Instrument Company Electronik recorder).

Procedures. The monkey is weighed and given 2 to 3 ml. Pentothal (12.5 mg. per ml.) by saphenous vein. The vein is then quickly dissected, cannulated with polyethylene tubing, and connected to a refillable 10 ml. syringe. Subsequent anesthesia is administered by this route. The monkey is kept covered on an electric heating pad.

Supplementary operative procedures, as needed, are performed on the mother. These have included cannulation of a carotid artery and establishment of a slow saline drip, tracheal cannulation, and catheterization of a uterine vein draining the primary placenta.

The uterus is exposed by a long mid-line incision. With the entire operating room completely darkened, the uterus is examined by special illumination procedures in order to visualize one or more of the interplacental vessels. Any or all of three procedures are followed. First, the bulbs of two small, bright pocket flashlights are placed against the uterus, and moved slowly and systematically over its surface (Fig. 2). It is necessary to visualize and identify the margins of the placenta. This structure remains dark whilst that part of the uterus which is adjacent to the bag of waters is translucent and becomes reddish colored. The systematic pattern of uterine vessels is observed and established with respect to the placentas. The translucent portion of the uterus is now examined carefully. Parts of the uterus overlying the amniotic fluid appear reddish when trans-illuminated. If necessary, the fetus may be rotated or shifted to bring new parts of the amniotic sac into view. Interplacental vessels, when visible, appear as straight or slightly curving vessels with no branching whatsoever. They appear to have indistinct margins (in contrast to the superficial uterine veins), since they lie deeper than any of the uterine vessels. Finally, when the uterus near an interplacental vessel is depressed with a finger, the vessel moves in a manner which is different from the more numerous and superficial uterine vessels. It is as if there were a parallax type of effect as the vessel, suspended in a small fold of membranes, moves in the fluid.

Of the nine attempts to cannulate an interplacental vessel, one (the eighth) was a failure, due to faulty judgment. The reasons for this are worth description. First, only one flashlight was employed. When exploring the uterus this failed to reveal a suspected interplacental vessel as well as two flashlights have been found to do. The intensity of illumination was only half as much, but more important, the shadow of what later was learned to be the vessel

FIG. 2. Method of illumination of gravid monkey uterus to show an interplacental artery connecting the primary with the secondary placenta. Note that it is uniformly large, unbranched, and runs a course different from the pattern of maternal uterine blood vessels.

sought for was not sufficiently distinct to permit its identification. Second, the placentas were separate but very close. Only two vessels traversed the uterine route, and these for only a short distance. When the cystoscope (see below) was employed, these vessels were seen clearly. Their appearance was misinterpreted because, for most of their distance, they ran together, but separated only near the secondary placenta. They appeared from outside the uterus, therefore, as a branching uterine vessel. When the uterine surface was examined in reflected light, large uterine veins draining the placenta were mistaken for the true interplacental vessels lying beneath. Since no other likely interplacental vessels were seen, it was wrongly concluded that we were dealing with another instance of contiguous primary and secondary placentas.

If the above steps fail to reveal an interplacental vessel, or if confirmation is required, a

strong narrow-beam operating light placed to one side of the operating table may be used. The beam is brought to bear upon the uterus. By moving the hand in the path of light, a small shadow may be cast upon the organ. The interplacental vessels stand out at the edge of the shadow as an elongation of it, since, lying in the light path, they cast their own shadow too, and may be seen when the light intensities are not too bright. This method is not regularly reliable. Should these two methods fail, resort may be made to the use of a small McCarthy child's cystoscope. This is employed in the following manner:

An area of uterus over the bag of waters is selected. A purse-string suture is passed in the uterine wall, in a circle about two centimeters in diameter. Two Allis clamps are attached to the uterus inside the circle and lifted by an assistant (Fig. 3). The uterine serosa between the clamps is snipped with fine scissors. With a scalpel in one hand and the lighted cystoscope in the other, a stab with the knife is made into the bag of waters. As the scalpel is withdrawn the cystoscope is slipped quickly into the bag of waters, the clamps are removed and simultaneously the purse-string is pulled tight. It is tied in a bow. With all room lights turned off, and the cystoscope light on, the uterus, in all areas adjacent to the bag of waters, yields a red glow wherever the bag of waters is illuminated. The interplacental vessels may now be seen as the cystoscope light is moved about. With luck, little probing is necessary, although at times considerable exploration may be required. As in the preceding method of visualization, the straight, unbranching character, the parallax effect, and the variance of direction of movement with the pattern of uterine vessels when the uterus is touched permit identification of the interplacental vessels. Having identified a vessel by any of the above three methods, the following steps are then taken, the uterus, meanwhile, being returned to the abdomen and nearly covered (see Fig. 3).

1. A clamp is placed in the uterine wall overlying the vessel where it is seen (Fig. 3). The room lights and operating light are again turned on.
2. The cystoscope (if used) is withdrawn, the hole being closed by retying the purse-string suture.
3. The uterine serosa is cut along the line of the vessel for about 5 mm. By spreading blunt forceps in this incision, the uterine opening is deepened and enlarged to about 8–10 mm. (Fig. 4).
4. As the membranes are reached, they protrude slightly, forming a sort of bleb (Fig. 4). The interplacental vessel may not be apparent at this juncture. Three or four Allis clamps are placed on the margins of the incision and elevated. The membranes under the edges of the incision are carefully stripped from the overlying endometrium by spreading the nose of a mosquito clamp between the membranes and uterus. By re-illuminating the tissue (in a darkened room), the interplacental vessel may be seen or it may be revealed by lifting or gently moving a small part of the membranes with a finger or a clamp. When the vessel is identified, it is moved into the opening.
5. Using a fine mosquito clamp, the chorionic membrane overlying the vessel is dissected bluntly by spreading it at one side of the vessel. The greatest care must be taken not to injure the amnion lying directly beneath the chorion. Experience suggests that the amnion is a tough but very thin membrane which does not yield readily to this dissecting maneuver since it is cushioned by liquid directly beneath. Great caution must be exercised.
6. The interplacental vessel will now lie naked, full of blood and sometimes pulsating. Ligatures may be passed under it, and cannulation made in the conventional manner (Fig. 5). We have taken pains to have the catheter point toward and reach the umbilical cord on the primary placenta (Fig. 6). In an early experiment, this was not done. Blood

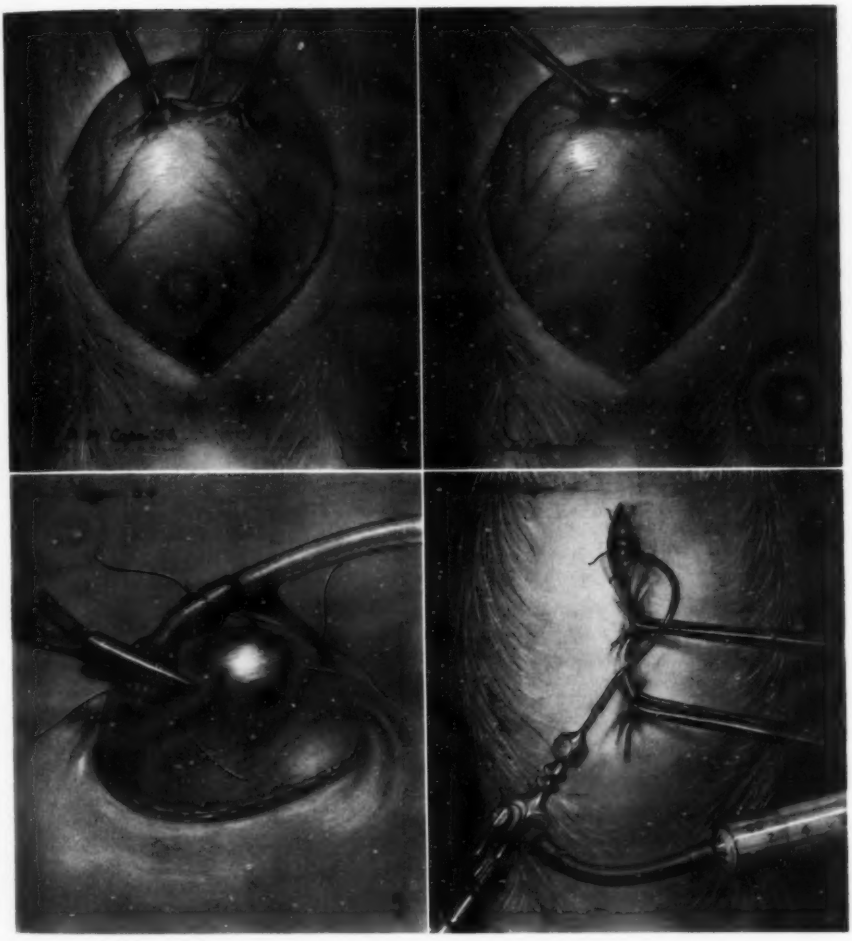


FIG. 3. Method of incising uterus above interplacental blood vessel.

FIG. 4. Exposure of the two underlying membranes (chorion and amnion) with the interplacental blood vessel between them.

FIG. 5. The interplacental vessel exposed, the chorion having just been dissected bluntly away. The method of cannulation is like that for other experimental techniques (i.e., carotid artery cannulation).

FIG. 6. The interplacental artery cannulated, with an in-lying plastic catheter. The uterine incision is closed by two interrupted sutures, and the abdomen is closed with clamps. Blood may be withdrawn from the fetal circulation with the fetus *in utero*; drugs or other substances injected, and blood pressure recorded by means of a strain gauge connected with the three-way stop-cock.

was obtainable, but with slightly more difficulty (i.e., at a slower rate) than in the remaining instances in which the connection was directly with the umbilical artery. In some cases, suction on the collecting syringe may not be used; in other cases, gentle suction may be applied. Usually, however, blood may be run directly into a collecting vial if the nature of the experiment permits this.

7. In order to prevent the small protrusion of amnion from undergoing enlargement during the experiment, the margins of the small incision may be closed by two to four interrupted sutures, care being taken to return gently the amnion to the uterus with the flat handle of a small pair of forceps.
8. In order to sample maternal placental blood at the time of sampling fetal blood, we have been able to catheterize one of the large, tributary uterine veins at the side of the uterus, below the ovarian pedicle, as mentioned above. In this way, blood samples may be taken from each side of a living placenta *in situ*.

RESULTS

Range of observations. A typical protocol of an experiment is given below to afford the reader an idea of the range of procedures and observations, that are possible when, with the fetus *in utero*, an interplacental vessel is cannulated. Here is shown the time, sampling of maternal and fetal bloods, injections into mother or fetus, and recording of fetal blood pressure under various conditions of study.

Protocol of Experiment

Monkey #939 (M-6)

Date: April 9, 1954
Weight: 5250 grams
Pregnancy: 145/168 days
Fetus: 380 grams
Placenta: 85 grams

Time	Interplacental Artery and Uterine Vein Cannulation		
	Pentothal Anesthesia (125 mg./cc.)	Samples of Blood	Other Procedures or Comments
9:30 a.m.		I. 6 cc. maternal blood (carotid)	
9:31	3.0 cc.		Saphenous vein cannulated
9:50	1.0 cc.		
9:51		II. 6 cc. maternal blood (carotid)	
10:10	1.0 cc.		Primary placenta identified.
10:21	1.5 cc.		Interplacental artery cannulated. (Later found to be $1\frac{1}{2}$ inches from umbilical cord).
10:40	1.0 cc.		
10:55	1.0 cc.		
11:17		III. 2 cc. maternal blood (carotid)	Record fetal blood pressure
		IV. 2 cc. fetal blood	Break in blood pressure record
11:20	1.0 cc.		
11:21–10			C^{14} glucose into fetus (interplacental artery)

Time	Interplacental Artery and Uterine Vein Cannulation		
	Pentothal Anesthfsia (125 mg./cc.)	Samples of Blood	Other Procecures or Comments
11:21–25		V. 2 cc. maternal blood	
11:22.0		VI. 2 cc. maternal blood	
11:23		VII. 2 cc. maternal blood	
11:21.40		VIII. 2 cc. maternal placental blood	
11:21.55		IX. 2 cc. maternal placental blood	
11:22.30		X. 2 cc. maternal placental blood	
11:22.30–11:23		XI. 2 cc. fetal blood	Record blood pressure
11:50	1.0 cc.		
12:13		XII. 6 cc. maternal blood (carotid)	Begin intravenous drip of 20% glucose (maternal)
12:16			
12:16.30			C^{14} glucose into fetus
12:16.45		XIII. 2 cc. maternal placental blood	
12:17.05		XIV. 2 cc. maternal placental blood	Record blood pressure
12:16.15–12:17.30		XV. maternal carotid blood	
12:18	1.0 cc.	XVIII. 2 cc. fetal blood	End intravenous glucose Record intravenous pressure
12:48			Record fetal blood pressure mother in distress; died. Inject 0.12 mgm. adrenaline into fetus
12:55			Fetus died.

Fetal Blood Pressure. Three examples of the effect on fetal blood pressure of uterine contraction and certain experimental modifications are shown in Figures 7, 8 and 9. All these observations were secondary to glucose-transfer studies in which repeated samplings of maternal and fetal bloods were made, depleting the fetal blood volume.

In Figure 7, the fact is demonstrated for the first time that as the uterus contracts and intrauterine pressure rises, the fetal blood pressure increases also by a nearly equal amount. During uterine diastole, fetal blood pressure was of the order of 40/32 mm. Hg. When the intrauterine pressure rose to about 25–30 mm. Hg, fetal blood pressure rose to 65 to 70 mm. Hg. There was a small decrease in pulse pressure simultaneously.

The effect of uterine systole upon (a) fetal heart rate, (b) venous return, (c) blood pressure, (d) placental blood flow, and (e) fetal reflexes may now be studied with the fetus *in utero*. Until the present time, this type of experimentation has not been possible.

Glucose placental transfer studies. The results of simultaneous sampling of fetal and maternal placental blood will be reported in detail elsewhere. Suffice it to say here that resting glucose and fructose levels in mother and fetus have been ascertained, during and after injection into the fetus, and into the mother under conditions of single rapid administration as well as under conditions of slow infusion over a long period of time. The fate of C^{14} glucose and of C^{14} fructose has likewise been determined under controlled conditions. It is evident from the limited experience at hand that the technic lends itself readily to a rather wide range of physiological studies of the fetus within its normal habitat inside the uterus. The results of these experiments will be reported subsequently.

Injection of drugs. Our experience with respect to blood pressure bears some further comment. We have, in addition to recording fetal blood pressure levels *in utero*, made observations with respect to a) injection of pitocin into the mother; b) injection of epinephrine (adrenal medulla extract) into the fetus; c) the effects of maternal death prior to the fetus, and d) the effect of fetal death prior to maternal death.

The injection of 2.5 units Pitocin intravenously into the mother caused a prompt uterine contraction which was sustained for many minutes (see Fig. 7). The fetal blood pressure rose to 60 mm. Hg, and then, with visible bradycardia showing on the recorder (but not readably recorded on the slowly moving paper) the blood pressure diminished to 40 mm. Hg at which level the pressure remained with minor changes for six or more minutes. There was then a gradual decline to about 30 mm. Hg.

At this point, 0.1 mg. epinephrine was injected into the fetal circulation. The heart rate became very fast, and pressure rose, as shown in Figure 7, to a height of more than 50 mm. Hg. However, the fast, rhythmic fluctuation

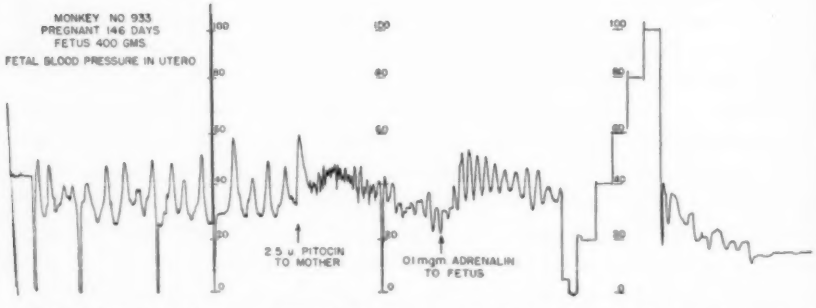


FIG. 7. Fetal blood pressure recorded *in utero*, amnion intact. Zero levels were determined six times. The large fluctuations in fetal blood pressure occurred synchronously with uterine contractions. See text for description of pitocin and epinephrine effects. Pressure indicated in millimeters of mercury.

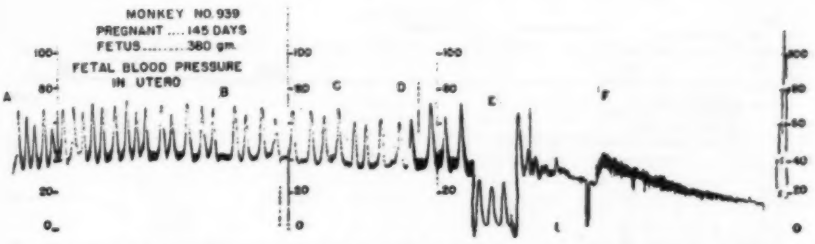


FIG. 8. Fetal blood pressure recorded *in utero*, amnion intact. Fluctuations with contractions as before. Pressure in millimeters of mercury. A, fetal and maternal blood samples taken; B, intravenous glucose begun to mother; C, 1 ml. Pentothal to mother; D, fetal and maternal blood samples; E, intrauterine (amniotic sac) pressure recorded; mother died at this time. F, 0.12 mg. epinephrine to fetus.

(three peaks per two minutes) in pressure suggests what we can not affirm positively from the record, namely, that the epinephrine injected into the fetus made the uterus contract and relax more extensively, on top of a waning pitocin-induced contraction. After calibration of the strain gage, which required 6 minutes, the fetal circulation was found to have declined, and the fetus died within the next eight minutes. The uterus was still firm and not indentable at this time. The fact that fetal blood pressure diminished suddenly at a critical point shows what has been suggested on hypothetical grounds, that at a critical level of intrauterine pressure, the blood pressure in the fetus diminishes. Up to this critical point, a differential of pressure is maintained between the intra- and extravascular compartments of the fetus over a rather wide range of normally fluctuating intrauterine pressures. The mechanism of fetal bradycardia has been studied experimentally (Reynolds, 1954) although now it needs to be reinvestigated more adequately *in utero*.

A second experiment (Fig. 8) showed the same fluctuations in fetal blood pressure. Pressure varied from about 40 to 60–65 mm. Hg. The additional observation was made that when the intrauterine pressure was recorded, it was found to be about 5 mm. Hg in uterine diastole and 30–35 mm. Hg during uterine systole. The fluctuation of 25 to 30 mm. Hg is the range of variation observed in fetal blood pressure. At the point marked B in Figure 8, glucose was given intravenously to the mother. At the point marked D, radioactive C^{14} glucose was injected into the fetus, washed in, and then samples of fetal and maternal blood were taken immediately and at certain intervals of time later. At this point, the mother began to show severe respiratory distress, after Pentothal administration. The uterus, judging from the fetal blood pressure record, contracted slowly and strongly. Fetal heart rate increased visibly and fetal pulse pressure increased substantially (4–6 mm. Hg to 8–10 mm. Hg). Intrauterine pressure was then recorded for about 9 minutes. Soon after this

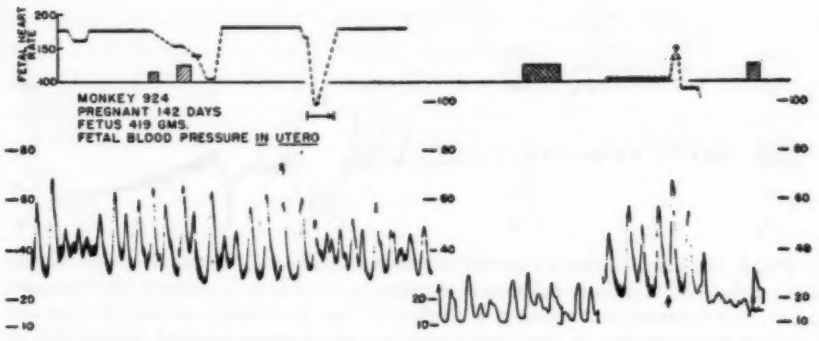


FIG. 9. Fetal blood pressure *in utero*, amnion intact. Fetal heart rate counted as record was made; graphed at top. Striped bars, 500 gm. weight and 1000 gm. weight placed on abdomen. Cross-hatched bar, 50 cc. of warm saline injected into amnion. Arrow, 50 cc. saline injected in amniotic sac. Intrauterine pressure recorded at that time. A lasting bradycardia noted, with cardiac irregularities at that time. 0.12 gm. of atropine injected into fetus caused momentary tachycardia. After fetus died, 1000 gm. weight was put on mother's abdomen. Rise in pressure compared with that seen when fetus was alive.

time it was found that the mother had died. Fetal heart rate and blood pressure commenced to fail. At this time, epinephrine was injected into the fetus. This drug caused a temporary increase in blood pressure and heart rate, but the response was short-lived. There was apparently no action of epinephrine upon the uterus, for no rhythmic contractions occurred following the injection of epinephrine. Probably none reached the uterus.

Active versus passive increase in uterine pressure. Figure 9 shows a third record of fetal blood pressure. This was made upon completion of an experiment using C^{13} and C^{14} glucose. This experiment had lasted for forty minutes, and involved withdrawal of a total of eight samples of fetal blood each of about 2 ml.

The first observation in this record was concerned with the effect upon fetal blood pressure and heart rate of increasing intrauterine pressure by placing 500 and 1000 gram weights on the abdomen. It will be seen that each weight, but especially the latter, caused an increase of fetal blood pressure (about 2–3 and 5–6 mm. Hg each). The heart rate was counted at frequent intervals.

The record shows that the addition of weight caused a bradycardia that was rapid in onset, and that this was rather marked with the heavier weight. Recovery was prompt. When weights were placed on the abdomen while intrauterine pressure was recorded, similar increases of intrauterine pressure were observed. The comparison with the effect on blood pressure is shown in Table I.

Injection of 50 cc. of warm physiological saline into the amniotic sac caused, (a) an increase in fetal blood pressure; (b) irregularity of uterine contractions

TABLE I

Changes in Fetal Blood Pressure and Heart Rate of Experimentally Increasing Intrauterine Pressure

Weights				Injection 50 cc. saline in amniotic sac.		
500 gms.		1000 gms.				
Heart Rate	Blood Pressure	Heart Rate	Blood Pressure	Heart Rate	Blood Pressure	Intrauterine pressure
beats/min.	*mm. Hg*	*beats/min.*	*mm. Hg*	*beats/min.*	*mm. Hg*	*mm. Hg*
−10	+2–3	−70	+5–6	−80	+4–5	+5

(as revealed by the records) and (c) prompt and profound fetal bradycardia (see arrow).

Clearly, passive increase of intrauterine pressure in either of these ways causes an increase in fetal blood pressure in the same manner as does active increase in pressure resulting from a uterine contraction. This point seemed worth establishing by observation, even though in a physical sense it had to be so. It was possible that a uterine contraction in the vicinity of placental attachment might have had a special vasopressor effect in the fetus through squeezing of the placental structure. This point needs more precise study, but it is now established that anything which augments intrauterine pressure also increases fetal blood pressure. When the pressure increase is sudden and marked by an addition of a kilogram weight or injection of fluid into the amniotic sac, the fetus exhibits bradycardia, as if to protect the fetus from too great or too sudden an increase in pressure. Decrease in fetal heart rate during uterine contractions at time of labor may serve a similar purpose.

SUMMARY

1. A method of catheterization of the fetal vascular system with the fetus *in utero* and amnion intact is described. The method involves insertion of a polyethylene tube into an interplacental artery of the monkey as it passes between primary and secondary placentas.

2. The method is applicable to animals having two or more discrete placental structures (e.g., monkey, sloth, sheep, goat, etc.).

3. Blood pressure and heart rate of the fetus *in utero* have been recorded. As the uterus contracts, fetal blood pressure rises, and as the former relaxes, the latter diminishes. Placing of weights on the abdomen causes increase in fetal blood pressure.

4. Bradycardia and blood pressure effects have been recorded under various conditions of stress, e.g., injection of pitocin into the mother, the placing of weight upon the uterus, and injection of warm saline into the amniotic sac.

5. Experiments with C^{12} and C^{14} glucose and fructose have been carried out in which injections have been made into mother and fetus, followed by frequent sampling of maternal (placental) and fetal blood. These experiments will be reported in separate publications.

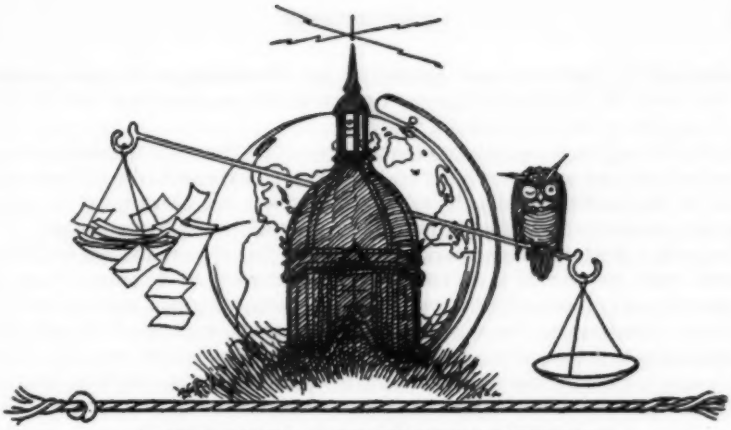
REFERENCES

ARDRAN, G. M., DAWES, G. S., PRICHARD, M. M. L., REYNOLDS, S. R. M. AND WYATT, D. G.: The effect of ventilation of the foetal lungs upon the pulmonary circulation. J. Physiol., 1952, **118:** 12.

BARCROFT, SIR JOSEPH: *Researches on Pre-Natal Life.* C. C. THOMAS, Springfield, Ill., 1947.

HUGGETT, A. ST. G.: Foetal blood gas tensions and gas transmission through the placenta. J. Physiol., 1927, **62:** 373.

REYNOLDS, S. R. M.: Homeostatic regulation of resting heart rate in fetal lambs. Am. J. Physiol., 1954, **176:** 162.



BOOK REVIEWS

(These reviews represent the individual opinions of the reviewers and not necessarily those of members of the Editorial Board of this Bulletin)

Metabolic Integrations. By P. G. WATSON. 15 pp., 4s. net. W. Heffer & Sons, Ltd., Cambridge, England.

Man's understanding of the natural world at any one time is made up of scattered facts and man's conceptual organization of them. These constitute a growing body of knowledge. Yet only a little is truly understood. All is in a state of flux and development, which we hope constitutes growth. Published material contains both facts and ideas or may emphasize either one. The present booklet consists of condensed ideas; these are chiefly put out in a highly organized fashion. Behind each of these ideas lies a variable amount of factual evidence of greater or lesser weight.

The contribution of this little 12-page booklet is to present in simple integrated patterns a large number of the more widely held ideas derived from experimental work in biochemistry. A whole textbook of biochemistry is represented in these concepts. The patterns constitute a framework of reactions which compose the biochemical activities of body cells. To the extent that they are correct they represent a step toward a goal which we must reach some day—a complete picture of cellular components and their interrelationships. This work is the kind of thing one must live with and refer to continuously if it is to become a part of him.

Now, while this type of achievement is needed, there is one big problem which arises with its creation. The farther we go with ideas at any level of factual knowledge the more tenuous the thread relating the ideas to fact; this cannot be helped. For this reason however, much that is written can be misleading, although sometimes stimulating. The situation is often found in the field of medicine. Some works are not very critically produced; others, a few only, are possibly too restricted to facts. The *Metabolic Integrations* by their aim must go pretty far from the solid ground of observation. They must be used, then, as suggestive, stimulating, not conclusive, even "heady" or dangerous ideas of how the cell in part "works". No evaluation is made in the booklet and so the danger areas are not posted nor are the more solid foundations indicated. This brave attempt may be a handicap, then, if misused. And it is hard to use it in a properly critical fashion.

F. W. B.

Antibiotika-Fibel. Indikation und Anwendung der Chemotherapeutika und Antibiotika. By ALBERT M. WALTER AND LUDWIG HEILMEYER. 813 pp., Ganzleinen DM 79 ($18.80). Georg Thieme Verlag, Stuttgart, Germany.

In their manual on the indications for and uses of antibiotic and chemotherapeutic agents, the authors have set out to provide in concise form a critical compilation of the knowledge extant in this rapidly expanding branch of pharmacology and therapeutics. To this end, they have produced an encyclopedic work within the confines of a single volume.

The book is divided into two principal sections. The first of these divisions comprises ten chapters which are devoted to the rationale of chemotherapy, to the pharmacology of the sulfonamides and of the antibiotics and to the laboratory techniques essential for the successful use of such agents. The second section of the manual, made up of thirteen chapters, is concerned with the infections and infestations of man and with their treatment. It begins with a survey of specific disorders which includes those of bacterial, rickettsial, viral, spirochaetal, fungal and protozoal origin and infections and infestations with parasites of higher orders. Much of the material is presented in tabular form, and data concerning incubation period, salient clinical features, laboratory diagnosis and active and passive immunization are included frequently in addition to the information on specific therapy. The remaining chapters deal with the treatment of infections of the several organ systems of the body and with the management of tuberculosis and of venereal disease.

Viewed as a whole, this volume contains an extraordinary amount of factual information. The material is well documented, the bibliographies are extensive and include many quite recent publications and the indices are adequate. It appears almost inevitable that, in a work of this kind, certain fine points of emphasis may at times seem lacking and that, in regard to certain details, one may differ with the authors. They have written, nonetheless, a most useful text and one which can serve as a ready source of reference to those with an interest in the subject of infectious disease.

ROBERT AUSTRIAN

Applied Pathology. As an Introduction to Disease and Its Control, 2nd edition. By CHARLES G. DARLINGTON AND CHARLOTTE F. DAVENPORT. 500 pp., $4.75. J. B. Lippincott Co., Philadelphia, Pa.

In this, the second edition of this volume, the authors integrate a background of pathology, sufficient for a course in nursing, with actual clinical nursing problems. The essential aspects of general and visceral pathology are covered in simple and concise form and the latest pathological concepts of disease processes are included. A bibliography is appended on every subject; this refers especially to pathology text-books and the nursing literature, rather than to experimental studies. In addition to an adequate correlation of the clinical picture found with the pathological process under discussion, many practical therapeutic nursing procedures are also included. A large portion of the volume is devoted to public health problems, microbiology and diagnostic laboratory procedures and interpretations. Biopsy and autopsy techniques are also described.

The authors obviously cater to the immature student. The material is presented in factual fashion omitting stimulating discussions on pathogenesis of disease or controversial aspects of etiology. Furthermore, the authors use the primary grade techniques of explanatory phonetic spelling as an aid to pronunciation of unfamiliar words as well as similar elementary review exercises. These teaching aids could surely be provided by a competent instructor. In this form, they only serve to detract from the otherwise excellent presentation of basic pathology for the instruction of the student nurse.

ELLA H. OPPENHEIMER

Funktionelle Röntgendiagnostik der Halswirbelsäule. By C. BUETTI-BÄUML. 160 pp., Ganzleinen DM 42. Georg Thieme Verlag, Stuttgart, Germany.

Prolonged post-traumatic symptoms referable to the neck are common notwithstanding negative routine roentgenograms. The author regards the conventional spine views suitable only for the detection of more obvious lesions and, therefore, he has attempted to develop radiologic methods to demonstrate otherwise unrecognized anatomic changes. He uses a special oblique view to demonstrate minimal changes in the apophyseal joints. The associated functional disturbances are demonstrated by flexion, extension, lateral flexion, and rotational studies.

The lesions of the cervical spine are subdivided into early degenerative and post-traumatic groups. Altered "resting position" of the cervical spine consists of straightening or minimal kyphosis or slight displacement of single segments. These changes are associated with definitely reduced or increased range of motion of the affected segments. The author has developed tables which show the normal range of extension and flexion as a base line to compare suspected abnormalities. Changes in function can be demonstrated in the absence of pathological changes such as osteo-arthritic spurs and narrowing of the intervertebral spaces. These functional changes are associated with abnormal strain on the cervical musculature and may be responsible for severe symptoms. In the post-traumatic group, the author has applied similar techniques.

The book is largely in atlas form and all of the illustrations are, as is the custom in Europe, positive reproductions of roentgenograms. Occasionally the detail described in the text cannot be clearly seen in the illustration. The book is valuable in that it points out that even minor changes in the cervical spine may be accompanied by disproportionately severe functional disturbances and symptoms. It is the impression of the reviewer that the same point could be made with something less than 158 pages.

GUNTER SCHULTZE

A. M. A. Fundamentals of Anesthesia, 3rd edition. Prepared under the Editorial Direction of the Consultant Committee for Revision of Fundamentals of Anesthesia, a publication of the Council on Pharmacy and Chemistry of the American Medical Association. 279 pp., $6.00. W. B. Saunders Co., Philadelphia, Pa.

As stated in the Preface, this excellent, concise text attempts "to set forth: (1) the basic principles of physiology, pharmacology, chemistry, and physics on which the sound practice of anesthesiology is based, (2) the technical procedures necessary to apply these principles effectively in relieving surgical pain, and (3) the application of these principles to the management of patients suffering from injury, illness, operation, or effects of depressant drugs."

Although, as is also pointed out in the Preface, its brevity necessitates a degree of dogmaticism in regard to many controversial problems in the field, its basic purposes are achieved remarkably well.

The text is well illustrated with simple, clear diagrams and pictures, and is accompanied by a good bibliography for the student interested in delving more deeply in specific problems.

It should prove a useful start for the beginning student in anesthesia, and a valuable adjunct for the teaching of this subject to the medical student.

The list of twenty-six authors involved in the preparation and revision of this edition contains some of the most eminent names in the field. It seems unfortunate that there is no way of identifying any portion of the text with an individual author.

DONALD F. PROCTOR

Mayo Clinic Diet Manual, 2nd edition. By Committee on Dietetics of the Mayo Clinic. 247 pp., $5.50. W. B. Saunders Co., Philadelphia, Pa.

This manual was written specifically for use in the Mayo Clinic and associated hospitals. It is a complete and concise collection of diets that should be of value to all physicians and dietitians as a reference work.

It will be particularly useful to physicians who do not have the services of a dietitian. Most diet therapists will not, however, be impressed with the provision of several standardized diets for each condition. For example, some physicians may be pleased to have four standard low calorie diets from eight hundred to fifteen hundred calories to hand out to patients. The diet therapist, however, will know that she will have more success if she designs her low calorie diets to suit the individual patient. It is also disappointing that the diabetic diets are not calculated with the new figures recently recommended by the American Diabetic Association to provide standardization of the calculations used in diabetic diets throughout the country.

Each diet in the manual is accompanied by a statement of composition and a comment on its adequacy as compared to the standards set up by the National Research Council. In cases in which the diets are below these standards in certain respects, there are recommendations as to the amount and kind of supplements needed. The appendix has many useful tables and lists such as height-weight-age tables, foods high in cholesterol, foods high in sodium and numerous others. The manual is well bound and indexed for constant use.

ELIZABETH CORNER

Psychology, the Nurse and the Patient. By DORIS M. ODLUM. 168 pp., $4.75. Philosophical Library, New York, N. Y.

This brief text is designed according to the author's foreword, to aid in the preparation of the undergraduate student of nursing in England for the examination in psychology for State Registered nurses there. Psychological concepts are presented in logical order with simplicity and informality of style. The emphasis is upon aiding the student nurse in developing insight into the emotional problems of general hospital patients and enabling her to become more skillful in applying sound psychological principles in her personal life. The text is eminently practical. Psychological concepts and principles are interpreted and their application demonstrated through the utilization of problems that arise recurrently in general hospital nursing. The text, while excellent for supplementary reading is too brief and too superficial for profitable use as a text in psychology by schools of nursing in the United States where undergraduate nurse preparation in that subject is thorough and extensive.

HARRIET MITCHELL

A Primer of Pulmonary Function. By HAROLD GUYON TRIMBLE AND JAMES KIERNAN. 22 pp. Printed through the cooperation of the California Tuberculosis and Health Association.

The authors of this small booklet have set as their object "to present a short summary of the basic pulmonary function tests, including a general outline of their techniques and their interpretations". Pulmonary function has been divided into two main categories of "Ventilation" and "Alveolo-Capillary Gas Exchange". The former is quite well discussed and includes detailed descriptions of vital capacity and maximal breathing capacity. However, perhaps a bit too much detail is given as to the method and equipment for performing the tests, with too little on the interpretation of the results. There is adequate discussion of combinations of measurements of resting and exercising ventilations, vital capacity and maximal breathing capacity as involved in the concepts of breathing reserve, Warring index

and air velocity index. Lung volumes are also fully described. However, insufficient emphasis is placed on the information which can be obtained about ventilatory function from adequate physical examination and fluoroscopy.

With regard to the aspect of "alveolo-capillary gas exchange", only an incomplete and inaccurate discussion of the blood gases is included. Oxygen is treated in some detail, including some apparently false concepts. However, carbon dioxide is very sketchily discussed. The extremely important concept of carbon dioxide retention and its recognition is almost completely ignored. Even the readily available measurement of carbon dioxide combining power has warranted only a passing word, and that not very clear or accurate. Similarly the discussion of blood pH is sketchy and in places misleading or erroneous, i.e. with regard to the decreased pH: "If hyperventilation is present, the acidosis is certain to be due to pulmonary disease rather than to depression of the respiratory center."

The aim of the authors in making available a "primer" of pulmonary function is worthwhile for such a discussion is generally lacking in the medical literature. But in some respects this seems a bit too detailed and in others, equally important, it is lacking or misleading. It does not succeed in making clear some very fundamental facts about pulmonary function as regards the handling of oxygen and carbon dioxide.

CAROL JOHNSON JOHNS

Reconstructive Surgery of the Eyelids, 2nd edition. By WENDALL L. HUGHES. 260 pp., $8.50. The C. V. Mosby Co., St. Louis, Missouri.

This book was first published in 1943 as a hundred and twenty-six page text with extensive illustration. Approximately one-hundred pages of text have been added, this having chiefly to do with reconstruction procedures devoted to the upper eyelid as well as some technical detail of the various methods of taking grafts. There is considerable historical detail on the development of reconstructive surgery and there are four-hundred and fifty-eight references listed in the back of the book, the majority of these dating before 1943. The book is profusely illustrated with a large number of drawings and photographs, most of which are lucid and well executed. There are a wide variety of problems presented and methods of reconstruction are discussed in individual case reports. This book is the type of work which should be present in all good reference libraries but is of interest chiefly only to the opthalmologists and plastic surgeons. It is an interesting, well presented and wide review of a difficult, narrow and very specialized field of surgery.

ROBERT W. JOHNSON, III

Diencephalon. Autonomic and Extrapyramidal Functions. By WALTER RUDOLF HESS. 79 pp., $4.00. Grune & Stratton, New York, N. Y.

This small monograph is the result of the desire of Professor Hess to acquaint a wider audience in the English speaking world with his extensive experiments upon the role of the diencephalon in the central nervous control of autonomic function, and of movement, which were originally described in Swiss and German publications. He also wishes to describe for that audience his technique for electrical stimulation of the brain in unanesthetized animals. In stating these as his objectives the author reveals a certain detachment from his public, for his works are already widely known and read, both in this country and in Great Britain, and the technique he has used so brilliantly has been current practice in laboratories in both countries for many years.

The experimental results described in this book are all drawn from material given in the two earlier monographs by the author: *Das Swishenhirn* (Benno Schwabe, Basle, 1949, and 2nd edition, 1954), and *Die functionelle Organisation des vegetativen Nervensystems*

(Benno Schwabe, Basle, 1948). The presentation here is too scanty to be of value to the investigator of brain function, who must consult the original works for useful information.

Some of the general hypotheses of central nervous function, which the author interweaves with occasional experimental support, are so controversial or so at variance with established fact as to be misleading to the general medical reader, or to the clinical neurologist. The general failure of this part of the book is due, in the opinion of the reviewer, to the fact that while experiments utilizing the technique of brain stimulation have yielded systematic information of great value in determining the *where* of brain function, they have failed to give reasonable answers to the question of the *how* of brain function. General hypotheses must be based upon the answers to this *how*, and the search for these answers is the principal challenge of modern neurophysiology.

VERNON B. MOUNTCASTLE

Proceedings of the Forum Sessions; Thirty-Ninth Clinical Congress of the American College of Surgeons, Chicago, October, 1953. I. S. RAVDIN, Chairman. 752 pp., $10.00. W. B. Saunders Co., Philadelphia, Pa.

Annual publication of the many significant papers read at the Surgical Forum sessions of the Clinical Congress of the American College of Surgeons is made possible by this book. These papers are selected from a larger group submitted to the Surgical Forum Committee and thus represent a valuable collection.

There are one hundred and thirty-eight separate papers, each about five pages in length. Their arrangement has much advantage over bound volumes of the standard surgical journals, in that all investigations relating to one organ system or subject are grouped together. Thus, a library containing the serial annual volumes of the Surgical Forum would offer opportunities for rapid and extensive reference to pertinent investigative work and its trends in many surgical fields.

This particular volume devotes approximately one-third of its pages to investigations relating to the heart, blood vessels, and circulation. Among these, explorations into techniques and methods of intracardiac surgery are prominent. Adequate space is given also to the many other fields of surgical endeavor. This book is indeed valuable, and in the words of I. S. Ravdin in his foreward, "Every surgeon who reads it will become the richer in his fund of surgical background."

DWIGHT C. MCGOON

Chemotherapy of Infections. By H. O. J. COLLIER with a Foreword by SIR ALEXANDER FLEMING. 248 pp., $4.00. John Wiley & Sons, Inc., New York, N. Y.

In this book the author emphasizes the fundamental significance of the reactions observed —both in parasite and in host—when there is an interaction between their cells and a chemotherapeutic agent. Based on the theory that the biological potency of a drug depends on its absorption on a receptor surface, and that such absorption is brought about by mutual attraction between chemical groups of the drug and the parasite, he points out that slight structural changes in the drug may "affect its fit with the receptor groups". Such concepts are pertinent when we consider the fact that some substances closely related chemically may exert quite different reactions when brought into contact with the micro-organisms. Under these circumstances, some such chemical substances have similar activity, while others may show reactions which are just the opposite. A good example of the latter is afforded by sulfanilamide and para-aminobenzoic acid, for those drugs though structurally much alike are antagonistic when brought into contact with susceptible bacteria.

These relationships between drugs and bacteria are further exemplified, as the author points out, by the "Woods-Fildes hypothesis" of bacterial metabolism and drug activity,

which includes the well-known theories having to do with growth factors, essential metabolites, and competitive inhibition of enzymes. Thus, in carrying out research for new chemotherapeutic substances, it is well to be guided by these fundamental principles.

The author's own field being that of pharmacology, this phase of drug therapy is given particular emphasis, with the inclusion of structural formulae of most of the important chemical and antibiotic compounds. The chief value of the work would seem to be in this category, and one might select the sections dealing with the sulfonamides, and those on the treatment of malaria and other tropical diseases as being of special interest. In contrast to this some of the chapters, for instance the ones concerned with the therapy of tuberculosis and those on penicillin, seem to fall somewhat short of this standard. These may be minor objections, however, for on the whole the book does contain a great deal of useful information.

A. M. FISHER

The Process of Psychotherapy. By HARRINGTON V. INGHAM AND LEONORE R. LOVE. 270 pp., $5.00. McGraw-Hill Book Co., New York, N. Y.

It takes considerable courage to attempt a description of how a psychotherapist works, which is the aim of this book. Much of psychotherapy proceeds through feelings and emotions rather than words and there is considerable disagreement about those parts that can be verbalized. Since the process is strongly influenced by the personality of the psychotherapist as well as his theories and training, no two psychotherapists operate precisely alike, and their descriptions of what they do may differ considerably from what actually takes place. Also, the results of psychotherapy have not yet been satisfactorily evaluated.

In the face of these difficulties the authors, a psychiatrist and a clinical psychologist, have managed to single out the generally agreed-upon concepts and methods of psychotherapy and to describe them in a simple and clear, though pedantic, fashion with a total absence of jargon.

The core of the book describes the relationship and insight gaining aspects of treatment from the standpoint of therapist and patient. The exposition is rounded out with two illustrative case presentations, one of a single interview to yield a microscopic view, and one of a complete course of treatment consisting of 36 interviews to afford a macroscopic picture.

To avoid controversial issues the authors have had to take refuge in generalities at times, and the use of an unqualified declarative style and a rigid outline occasionally implies more certainty than actually exists. It is, however, heartening to discover how much can be said of a specific nature which all psychotherapists would accept.

On the whole, the authors have performed an admirable job and this book can be warmly recommended to all those who have been waiting for a simple statement in plain English of what psychotherapy is about.

JEROME D. FRANK

BOOKS RECEIVED FOR REVIEW

The Concept of Schizophrenia. By W. F. MCAULEY. With a Foreword by JOHN H. EWEN. 145 pp., $3.75. Philosophical Library, New York, N. Y.

Human Relations in Action. By H. EDMUND BULLIS AND CORDELIA W. KELLY. 86 pp., $1.50. G. P. Putnam's Sons, New York, N. Y.

Metabolic Interrelations. With Special Reference to Calcium. Transactions of the Fifth Conference, January 5 and 6, 1953. Edited by Edward C. Reifenstein, Jr. 386 pp., $5.00. The Josiah Macy, Jr. Foundation, New York, N. Y.

Of Publishing Scientific Papers. By GEORGE E. BURCH. 40 pp., $2.75. Grune & Stratton, New York, N. Y.

Principles of Occupational Therapy, 2nd edition. Edited by Helen S. Willard and Clare S. Spackman. 376 pp., $5.50. J. B. Lippincott Co., Philadelphia, Pa.

Recent Developments in Psychosomatic Medicine. Edited by Eric D. Wittkower and R. A. Cleghorn. 495 pp., $10.00. J. B. Lippincott Co., Philadelphia, Pa.

Shock and Circulatory Homeostasis. Transactions of the Third Conference, September 14, 15 and 16, 1953. Edited by Harold D. Green. 230 pp., $3.50. The Josiah Macy, Jr. Foundation, New York, N. Y.

Teaching in the OutPatient Department. By SISTER MARY ISIDORE LENNON. 239 pp., $4.00. G. P. Putnam's Sons, New York, N. Y.

Textbook of Pediatrics, 6th edition. Edited by Waldo E. Nelson. With the Collaboration of Seventy Contributors. 1581 pp., $15.00. W. B. Saunders Co., Philadelphia, Pa.

Transactions of the 13th Conference on the Chemotherapy of Tuberculosis. Held on February 8 to 11, 1954. St. Louis Medical Society. St. Louis, Missouri by the Veterans Administration: Army: Navy: Prepared and Edited by the Veterans Administration Area Medical Office, St. Louis, Missouri. 441 pp.